

Structure-function analysis of staphylococcal inhibitors of neutrophil granule enzymes

by

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B.Pharm., University of Pune, 2013

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Department of Biochemistry and Molecular Biophysics
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Abstract

Neutrophils are the most numerous leukocytes in humans and serve as the first responders to bacterial infection. They combat pathogens through phagocytosis, degranulation, and release of neutrophil extracellular traps (NETs). Upon phagocytosis of bacteria, neutrophils mobilize their granule-resident defense systems which include the antibacterial enzymes myeloperoxidase (MPO) and the neutrophil serine proteases (NSPs) neutrophil elastase (NE), proteinase-3 (PR3) and cathepsin-G (CG). Whereas MPO uses H_2O_2 as a precursor for production of cytotoxic hypohalous acids, neutrophil serine proteases exhibit broad substrate specificity, enabling them to cleave multiple bacterial proteins. The presence of these antibacterial defense systems allows neutrophils to serve as an effective defender against pathogens. However, due to extensive selective pressure, certain pathogens like *Staphylococcus aureus* have evolved the molecular means to evade neutrophil antibacterial defenses. In this dissertation, we explored the structure and function of two different families of staphylococcal proteins that inhibit neutrophil antibacterial enzymes.

Staphylococcal Peroxidase Inhibitor (SPIN) is an ~8 kDa immune evasion protein that binds MPO with high affinity, inhibiting its activity. Structure-function investigations have revealed that SPIN acts as a molecular plug, blocking solute exchange with the MPO active site and thereby inhibiting production of hypohalous acids (e.g. HOCl, HOBr, etc.). The triple-helical C-terminus of SPIN mediates MPO binding, while its N-terminus is required for MPO inhibition. The SPIN/MPO co-crystal structure shows the SPIN N-terminus adopts a β -hairpin conformation in the presence of MPO. However, the lack of intrinsic stabilizing features within the SPIN N-terminus suggests it adopts a disordered state before binding to MPO. To investigate this further, we introduced a disulfide to trap the SPIN N-terminus in the MPO-bound conformation, resulting

in a variant named SPIN-cys. We used a combination of 2D and 3D solution NMR experiments to confirm the presence of the disulfide bond and investigate the solution structure features. Our analysis revealed that while SPIN-cys adopts the characteristic triple helical structure at its C-terminus, the N-terminus remains disordered in the solution. Further examination of backbone dynamics through HETNOE and NMR relaxation experiments showed that the incorporation of a disulfide bond to the N-terminus of the protein increased its stability compared to the wild-type SPIN. SPR analysis showed that SPIN-cys has a higher affinity for MPO than its wild-type counterpart, resulting in enhanced inhibition of MPO. It appears that the disulfide bond limits the range of conformations in the N-terminus of SPIN, which is responsible for the increase in affinity and apparent enhancement of inhibition.

The multidomain extracellular adherence protein (Eap) and its single-domain homologs (EapH1 and EapH2) are potent inhibitors of NSPs. While EapH1 employs a globally similar binding mode to inhibit CG and NE, understanding NSP inhibition by EapH2 has been challenging due to the absence of NSP/EapH2 cocrystal structures. To address this gap, we utilized NMR chemical shift perturbation (CSP) and crystallography techniques to investigate EapH2 and its interactions with CG and NE. We discovered that while overlapping regions of EapH1 and EapH2 were involved in CG binding, distinct regions of EapH1 and EapH2 experienced changes upon NE binding. This suggested that EapH2 may be capable of simultaneously binding and inhibiting CG and NE. We confirmed this unexpected finding by solving crystal structures of the CG/EapH2/NE complex and validating their functional relevance through enzyme inhibition assays.

Thereafter, we investigated NSP binding and inhibition by both the individual domains that comprise Eap as well as full-length Eap itself. Crystallography analysis showed that individual Eap domains inhibit CG and NE simultaneously, similarly to EapH2. Since full length Eap exists

as a multidomain protein in its natural state, we explored the simultaneous occupancies by NSPs at its respective sites using SAXS. These results revealed that Eap can simultaneously bind four CG molecules or four NE molecules in solution. Furthermore, Eap34, a multi-domain variant, demonstrated the ability to occupy all binding sites simultaneously, and formed higher-order structures in the presence of NSPs. These observations strongly suggest that the full-length Eap protein is a polyvalent and bifunctional inhibitor of neutrophil serine proteases.

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Dedication

To my beloved mother, Shrimati Manisha, for her steadfast encouragement in pursuing my dreams.

To my dear father, Shri Bhalchandra Mishra, for his unwavering care and boundless support towards my passions and endeavors.

Thank you so much for your love and support; this work wouldn't have been completed without you.

Chapter 1 - Introduction

Neutrophil development and recruitment

Neutrophils, the most abundant circulating leukocytes, are short-lived granulocytes that serve as the body's initial defense against invading microbes (1). They are derived from pluripotent hematopoietic stem cells and consist of multi-lobed nucleus (2, 3). During their differentiation process, neutrophils acquire phagocytic ability followed by oxygen-dependent microbicidal activity, enhanced adhesiveness, motility, chemotactic responsiveness, and other cell-specific traits (4–6). This progression occurs through a carefully regulated series of steps leading to mature neutrophils. As neutrophils mature, the number of mitochondria and ribosomes decreases, while glycogen granules, the primary energy source, accumulate in the cytoplasm (2, 7). The maturation process also involves the filling of primary/azurophilic granules and secondary granules in the cytoplasm of mature neutrophils. Azurophilic granules contain myeloperoxidase (MPO), neutrophil serine proteases (NSPs) like cathepsins, proteinase-3, elastase, and other antimicrobial proteins, while secondary granules contain lactoferrin, human neutrophil lipocalin (HNL), metalloproteinases, and others (2, 8–12). Mature neutrophils, upon entering the bloodstream, circulate for 10-24 hours before migrating to tissues where they function for approximately 2 days before undergoing apoptosis, typically initiated by macrophages (2, 3, 13–16).

A dynamic subset of neutrophils actively patrols the system, seeking out signs of tissue damage, inflammation, or invading pathogens. When inflammation occurs or pathogens are detected, various cells like mast cells, macrophages, etc., release inflammatory mediators that bind to specific receptors on neutrophils (17–20). This interaction leads to the recruitment of neutrophils to the sites of infection.

Pathogen recognition and neutralization

Once recruited to the site of infection the neutrophils bind and ingest the invading pathogen by phagocytosis process. Bacteria consist of conserved pathogen-associated molecular patterns (PAMPs) which can be directly recognized by the neutrophil surface pattern recognition receptors (PRR). Such receptor engagement activates signal transduction which later facilitates activities like phagocytosis and degranulation (21).

Phagocytosis entails the ingestion of microbes into the isolated phagosomal compartment of neutrophils, which is laden with a diverse array of toxic antimicrobials. As the phagosomes of neutrophils mature, the ingested microbes are transported into membrane-bound phagosomal structures, ultimately leading to bacterial death (22). Concurrently, during phagosomal maturation, neutrophil secretory vesicles and granules fuse with the phagosome, enriching it with components capable of microbial destruction. This process includes the incorporation of the transmembrane component of NADPH oxidase, which becomes active later (23–26). Active NADPH oxidases generate superoxide from O_2 , subsequently converted to H_2O_2 . MPO, abundant in primary granules, catalyzes the production of highly bactericidal hypohalous acids (e.g. HOCl) using H_2O_2 as a substrate (27–30). The fusion of azurophilic granules with the phagosomes further enhances their oxidative killing capability (31).

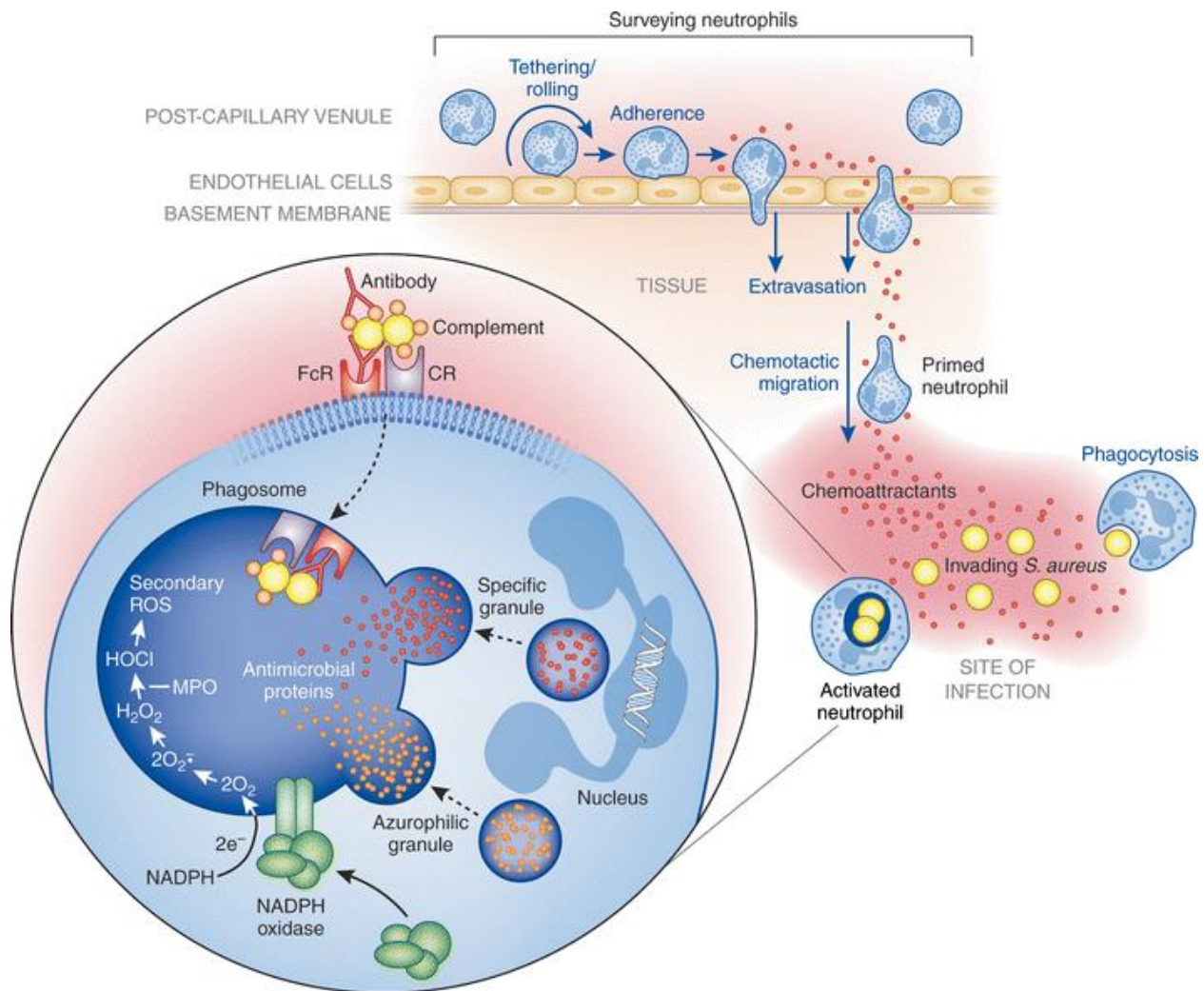


Figure 1.1 Migration of neutrophils from vascular space to infection site during microbial killing.

Neutrophils exit blood vessels, migrate to infection sites, and engulf and kill microbes. They patrol post-capillary venules for signs of infection, interacting briefly with endothelial cells. This interaction allows them to roll along the vessel wall and search for signs of infection. Chemotactic molecules released from the infection site bind to neutrophil receptors, promoting firm attachment to the vessel wall. Firm attachment enables neutrophils to migrate through the vessel wall (extravasation) into the tissue. Once in the tissue, they move toward the infection site, where they engulf and destroy microbes using reactive oxygen species and antimicrobial proteins (31).

In addition to MPO, NSPs are significant constituents of azurophilic granules, which are released into the phagosome during maturation. These NSPs, including cathepsin-G (CG), proteinase-3 (PR3), and neutrophil elastase (NE), are tightly bound to proteoglycans in azurophilic granules and become active only upon release into the phagosome (32–36). Belonging to the

chymotrypsin family of serine proteases, they possess the Ser-His-Asp catalytic triad at their active site. Despite sharing 35-56% sequence identity and having highly similar structures, they exhibit a diverse substrate specificity, enabling cleavage of a wide range of substrates to enhance bactericidal activity within the phagosome (37, 38). Moreover, besides oxidative killing, neutrophils can undergo degranulation to release their granular content into the extracellular milieu, further contributing to pathogen eradication outside the cell (1).

In addition to phagocytosis and degranulation, neutrophils can undergo a process called neutrophil extracellular trap (NET) formation, where they release web-like structures composed of antimicrobial proteins attached to the DNA backbone. These NET structures contain approximately 30 proteins, including histones, MPO, NSPs, etc., which work together to eliminate trapped bacteria (39). Classical NETosis, a form of NET formation, involves the release of NE from cytoplasmic granules, which cleaves histone linkers, leading to chromatin decondensation. Subsequently, MPO enters the nucleus to further facilitate nuclear DNA decondensation. The nuclear envelope then breaks down, releasing decondensed nuclear chromatin into the cytoplasm, where it binds with cytoplasmic and granular components. Within 3-8 hours, the activated neutrophil's membrane ruptures, releasing NETs and resulting in cell death (40–45). In addition to classical NETosis, a non-suicidal form called vital NETosis has also been identified (46). Neutrophils have been observed releasing NETs while actively crawling, without undergoing lysis. Subsequently, these neutrophils were found to remain alive and capable of continuing their functions, including chemotaxis, phagocytosis, and bacterial killing, after releasing the NETs (39, 47, 48). Additionally, neutrophils can release mitochondrial DNA independently of cell death, representing a distinct type of NETosis (49).

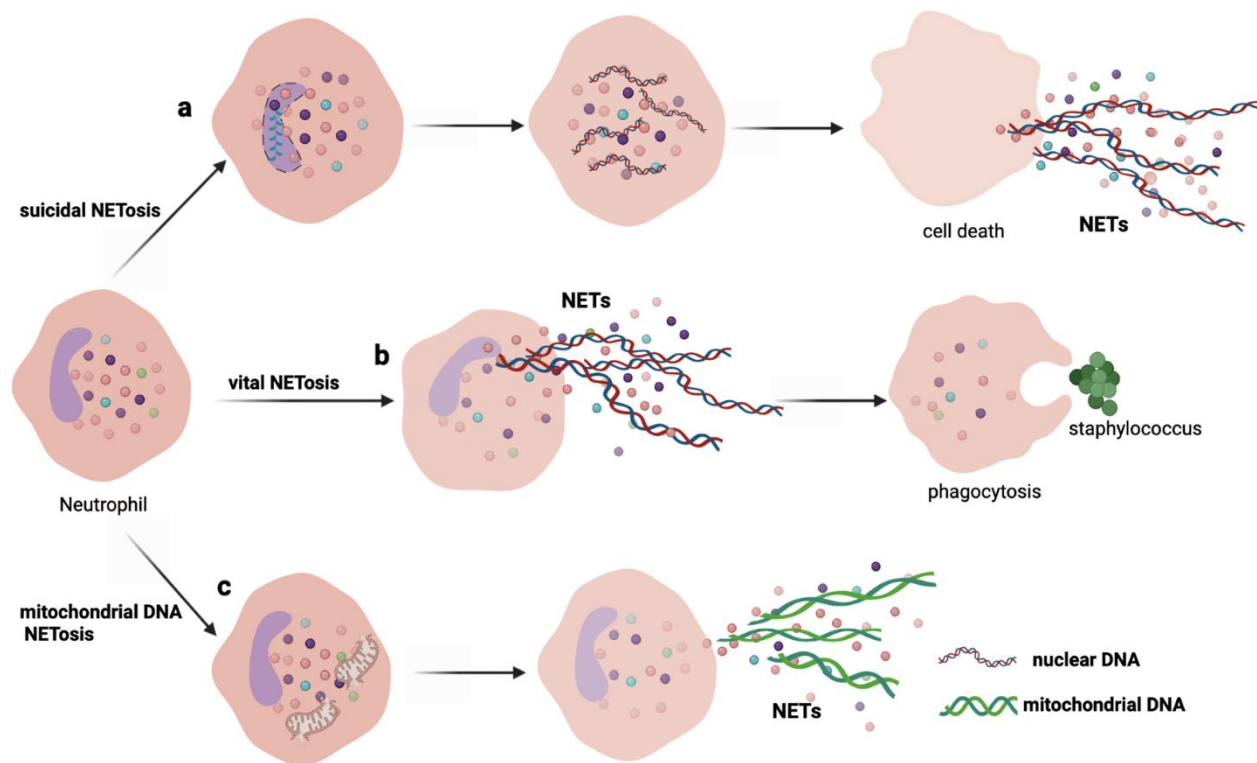


Figure 1.2 Types of NET formation.

a. The classical form of NETosis is suicidal NET formation, featuring nuclear chromatin decondensation, NET release, membrane rupture, and cell death. b. After releasing NETs the neutrophils can remain alive and remain phagocytic is the vital NETosis. c. The generation of NETs using mitochondrial DNA rather than nuclear DNA, is mitochondrial DNA NETosis (50).

Possessing these arms, the neutrophils present a tailored response against invading pathogens which makes them an efficient pathogen executioner. However, due to the long interplay between the invading pathogens and neutrophils, there are certain pathogens, such as *S. aureus* adapted themselves to evade neutrophil-based killing mechanisms (51). *S. aureus* has been shown to remain alive inside the phagosome (52, 53). Given that the neutrophils express a wide array of arms to kill the invading pathogens, *S. aureus* being able to stay alive inside the phagosome must be coming from a range of evading mechanisms (54–56). Extensive research on *S. aureus* with the aim to identify and characterize proteins important for the survival against host innate immunity resulted in the identification of numerous staphylococcal proteins, called immune

evasion proteins. In this dissertation, we will focus on staphylococcal immune evasion protein SPIN, Eap, and Eap homologs.

SPIN: a potent MPO inhibitor

de Jong et. al. (2017) discovered a *S. aureus* immune evasion protein capable of inhibiting the production of hypohalous acids from MPO called Staphylococcal Peroxidase INhibitor (SPIN). Since MPO is a major component of the phagosome and NETs, inhibiting it will provide bacteria a significant advantage against neutrophils. SPIN is a 73-residue protein, ~8.3 kDa, which binds to MPO with a high affinity (~10 nM), measured by SPR, and inhibits the peroxidase activity of MPO with an IC₅₀ of ~ 7 nM. Subsequent protein crystal analysis using co-crystal structure of SPIN/MPO at 2.4 Å limiting resolution revealed that the protein is comprised of a triple helical structure with a small β-hairpin (~10 residues) at the SPIN N-terminus. The detailed complex structure analysis revealed that upon binding to MPO, the β-hairpin structure can completely occlude the substrate access to the catalytic heme (56). The NMR analysis using the unbound SPIN protein revealed that the N-terminal β-hairpin remains disordered in the unbound state. The TALOS-N secondary structure prediction suite revealed similar characteristics like that observed in crystallography. It showed a high possibility of three-helix structures consistent with the bound SPIN crystal structure. The NMR relaxation measurement studies revealed that the average R₂/R₁ was higher for the C-terminus of the protein (3.72 ±0.73) compared to the N-terminus (2.76 ±0.59), indicative of flexibility in the N-terminus of SPIN (57). Chapter 2 will provide a detailed review on the SPIN protein. Since the N-terminus lacks any intrinsic stabilizing features, such as a disulfide, the flexible N-terminus is an expected feature. In order to further study the effect of the SPIN N-terminus on binding and inhibition of MPO we introduced a disulfide in the N-terminus to physically constrain the N-terminus flexibility. We found that the introduction of the disulfide

to the N-terminus has increased the affinity and inhibition of SPIN towards MPO by the factor of 2.5. A detailed explanation of the outcomes can be found in chapter 3.

Eap: The serine protease inhibitor

Given the indispensable role of NSPs in the phagosome killing *S. aureus* survivability inside the phagosome hints the plausibility of NSP inhibition by *S. aureus*. Stapels et. al. (2014) discovered a unique class of proteins capable of potently and specifically inhibiting NSPs noncovalently, called extracellular adherence proteins (Eap). The Eap family comprises three proteins, single domains Eap homologs EapH1 and EapH2 and a multidomain Eap protein. Eap can have 4-6 repeat domains depending on the strain of *S. aureus*. Each Eap domain consists of ~100 residues and the domains show high structural homology (55). Although the multidomain Eap protein displays multiple functions including inhibition of classical and lectin complement pathways, single domains are sufficient for NSP inhibition (55). The structure of Eap homologs and Eap domains has been explored in depth using protein crystallography and NMR. Structural explorations have revealed that Eap domain proteins adopt a β grasp fold comprising a large helix structure diagonally superimposed on a five-stranded β -sheet (58–62). The details on the structural features of Eap proteins can be found in chapters 4, 5, 6, and 7.

The NSP interaction with the Eap homolog, EapH1, has been extensively explored. EapH1 binds and inhibits both CG and NE through a globally similar binding mode. Co-crystal structure analysis with EapH1/CG and EapH1/NE revealed that the protease inhibitor binds both the NSPs near its active site. These interactions are highly complementary in nature, one can expect the complete hindrance of substrate access to the active site of NSPs. The EapH1 binding site undergoes substantial change as the B-factor near the EapH1 binding site undergoes a major change upon binding to NSPs (55).

The detailed understanding of EapH2 interactions with the NSPs has become evident very recently. Structural analysis using NMR chemical shift perturbation (CSP) revealed that EapH2 can bind to CG through the site similar to EapH1 however the NE binding site is altogether different. This feature was further explored by solving the crystal structure of CG/EapH2/NE ternary complex structure. Detailed analysis of the crystal structure revealed that the protease inhibitor binds to the active site of both the proteases simultaneously (63). A detailed explanation of how EapH2 inhibits both the NSPs simultaneously can be found in Chapter 6.

Gido et al. (2023) recently detailed the NSP inhibition by Eap domain 1 and domain 2. Co-crystal structure analysis of Eap1/CG and Eap2/CG showed that both domains bind to the CG similarly to EapH1, while NE binding is analogous to EapH2 (64). This suggests that, like EapH2, Eap domain 1 and 2 might be capable of simultaneously inhibiting both the proteases. Our understanding of NSP inhibition by Eap domains and Eap full length was further improved using protein crystallography and SAXS. Crystal structure analysis revealed that all the Eap domain proteins bind to serine proteases similarly to EapH2. SAXS analysis showed that Eap can act as a polyvalent inhibitor of NSPs by inhibiting multiple NSPs simultaneously. We also noticed that multidomain Eap bound to NSPs exhibits a tendency for oligomerization. This phenomenon might assist bacteria in either inhibiting NET formation, rendering NET more susceptible to bacterial DNase, or both. However, further analysis is necessary to support this claim. A detailed understanding on this can be found in Chapter 7.

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Chapter 2 - Structure, Function, and Mechanistic Insights into A Novel Family of Myeloperoxidase Inhibitory Proteins Expressed by Staphylococci

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Abstract (200 words)

The Gram-positive pathogen *Staphylococcus aureus* has emerged as a model system for understanding the process of innate immune evasion, particularly as it pertains to the complement/neutrophil axis that drives opsonization and bacterial killing within the phagosome. Although initial work focused on staphylococcal molecules that disrupt the extracellular complement system, more contemporary studies have identified evasion proteins that primarily function inside the phagosome where they inhibit key enzymes of the neutrophil anti-bacterial arsenal. One such molecule is the Staphylococcal Peroxidase Inhibitor (SPIN). *S. aureus* SPIN is a small, secreted protein whose expression is upregulated following phagocytosis. SPIN binds with low-nanomolar affinity to human myeloperoxidase (MPO), potently inhibits MPO activity, and appears to promote survival of *S. aureus* within the neutrophil phagosome. Structural studies involving solution nuclear magnetic resonance spectroscopy and X-ray crystallography have provided insights into how SPIN inhibits MPO. This has led to a model where SPIN acts as a molecular plug and blocks solute exchange with the MPO active site. The recent identification of SPIN homologs from other staphylococci has allowed for comparative structural and functional studies between these proteins. This chapter summarizes the results that have led to current understanding of this novel family of staphylococcal MPO inhibitors.

Introduction

Neutrophils are the earliest acting cellular components of the human innate immune system. Though they serve many roles in defense and repair, they are best known for their ability to engulf and kill invading microorganisms. Neutrophils rapidly sense and respond to pathogen-derived molecular patterns (e.g. formylated peptides), as well as danger or damage-associated (e.g. C5a) molecular patterns through a series of high-affinity receptors exposed on their plasma membrane. This allows neutrophils to be the first leukocytes to infiltrate sites of nascent infection or damaged tissues in the process of healing (1-4). Activation of neutrophils results in remarkable morphological changes. It also triggers mobilization and secretion of their cytosolic granules (5). Chief among these are the azurophilic granules, which contain critical components of the neutrophil anti-bacterial arsenal. Two of the most abundant components of azurophilic granules are the enzyme myeloperoxidase (MPO), which converts hydrogen peroxide (H_2O_2) into cytotoxic hypohalous acids (e.g. HOCl), and a series of chymotrypsin-like serine proteases, called Neutrophil Serine Proteases (NSPs), which can directly attack the pathogen cell, its contents, or secreted products (6, 7). This concerted action of MPO and NSPs forms the foundation of neutrophil-mediated defense against invading bacteria.

Since they are immediately confronted by the host innate immune system, there is strong selective pressure for invading bacteria to evolve the molecular means to disrupt the host immune system or escape it altogether. Although studies on various pathogens have identified a diverse array of these so-called immune evasion strategies (8, 9), the Gram-positive bacterium *Staphylococcus aureus* is particularly noteworthy for the sheer number of different proteins it produces to interfere with opsonization and phagocytosis (8-12). Some studies have also shown that a population of *S. aureus* cells are resistant to killing within the phagosome (13). Given the

biochemical complexity of the phagosome and the extreme environment it presents (14-17), the fact that *S. aureus* can survive there at all implies that its physiological changes must be multifaceted. It also suggests that the components of the staphylococcal innate immune evasion program that act within the phagosome must be somewhat protective. Indeed, the recent identification of a potent family of secreted NSP inhibitors that promote *S. aureus* virulence is consistent with this premise [15]. Nevertheless, this also raises questions of whether *S. aureus* targets other components of the neutrophil anti-bacterial arsenal that act primarily within the phagosome.

SPIN: A Novel Myeloperoxidase Inhibitory Protein Expressed by *Staphylococcus aureus*

MPO serves a central role in the innate defense against pathogens (14-17). It therefore represents an extremely attractive target for elaboration of an *S. aureus* innate immune evasion strategy. Since no MPO-inhibitory evasion protein had yet been identified, a secretome phage display library from *S. aureus* strain Newman was probed for proteins that bound to human neutrophil degranulate fluid (18, 19), of which MPO is a predominant constituent. A single, highly enriched clone representing the gene NWMN_0402 was isolated following several rounds of enrichment (19). NWMN_0402 clone encodes a pre-protein of 105 residues that contains a 32-residue signal peptide, leaving a predicted matured protein of 73 residues and a molecular mass of 8,380 Da. A recombinant form of NWMN_0402 protein bound specifically to immobilized human MPO in ELISA studies. Subsequent surface plasmon resonance experiments revealed an equilibrium dissociation constant of 10 ± 0.6 nM, with association and dissociation rates constants on the order of $10^5 \text{ M}^{-1}\text{s}^{-1}$ and 10^{-3} s^{-1} , respectively (19). Significantly, this same protein inhibited the peroxidase activity of MPO with an IC_{50} value of approximately 7 nM (19). This value was in excellent agreement with the affinity measured by SPR, strongly suggesting that NWMN_0402

might act as a competitive inhibitor of MPO. Consequently, NWMN_0402 was renamed “SPIN” for Staphylococcal Peroxidase Inhibitor.

The SPIN protein has no obvious sequence homology to other characterized proteins (19). This was a major impediment to developing hypotheses concerning its mechanism of MPO inhibition. To address this limitation, SPIN was crystallized bound to a recombinant form of human MPO (hereafter rhMPO) (19). The SPIN/rhMPO structure was solved to 2.4 Å limiting resolution and refined to $R_{\text{work}}/R_{\text{free}}$ values of 18.4% and 24.1%, respectively (19). This structure revealed that SPIN was comprised of a peculiar β -hairpin at the SPIN N-terminus, followed by a compact α -helix bundle domain that is common among staphylococcal innate immune evasion proteins (19, 20), including Efb-C (21), Ehp (22), and SCINs (23, 24). Since the ~10-residue β -hairpin lacked any intrinsically stabilizing features of its own, it was predicted to be disordered prior to binding MPO (19). This turned out to be the case, as will be further described in a separate section of this text.

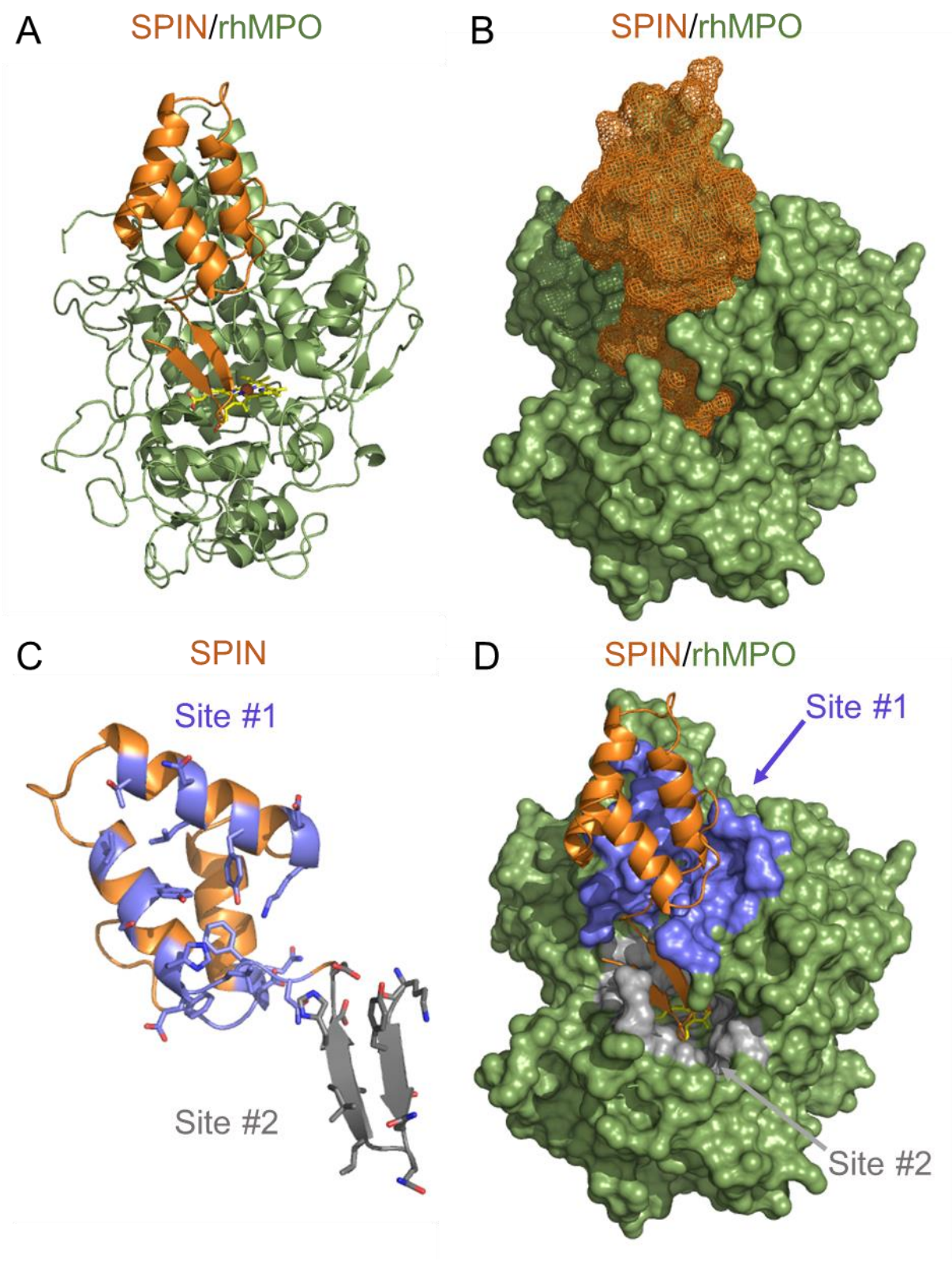


Figure 2.1 Structure of the Staphylococcal Peroxidase Inhibitor, SPIN, bound to a recombinant form of human MPO.

(A) Ribbon representation of the final model for *S. aureus* SPIN (orange) bound to a recombinant form of human MPO (olive), as rendered from the PDB entry 5UZU (19). The location of the heme prosthetic group (drawn as yellow sticks) was inferred from PDB entry 1CXP (25). (B) Representation of the SPIN/rhMPO structure where SPIN is drawn as an orange space-filling mesh and rhMPO is an olive molecular surface. Note the complete occlusion of access to the catalytic heme by the N-terminal β -hairpin of SPIN. This feature is a key determinant of MPO inhibition by SPIN. (C) Representation of the *S. aureus* SPIN protein illustrating the nature of the two distinct rhMPO interfaces. Interface #1 (labeled “Site #1”) is comprised of residues within the α -helical bundle domain and is colored light purple. Interface #2 (labeled “Site #2”) is comprised of residues within the β -hairpin and is colored silver. The sidechains of residues that form contacts with rhMPO in the structure are shown as ball-and-stick models. (D) Representation of the SPIN/rhMPO structure where the two distinct SPIN-binding surfaces on MPO are colored according to their participation in either Interface #1 or Interface #2.

Examination of the SPIN/rhMPO structure revealed two distinct interfaces that contribute to complex formation (19). The first interface involves the SPIN α -helical bundle domain and lies at the side of the MPO active site channel entrance. This interface buries $726 \text{ \AA}^2$ of the inhibitor, or approximately 45% of the $1,633 \text{ \AA}^2$ of SPIN surface area buried in the complex. The second interface involves the SPIN β -hairpin, which lies entirely within the MPO active site channel. This interface buries $907 \text{ \AA}^2$, or approximately 55% of the total SPIN surface area buried in the complex. For reasons that remain unclear, the heme occupancy of the rhMPO in this structure was negligible (19); however, it was possible to infer the location of the heme prosthetic group using the structure of native human MPO as a guide (25). This indicated that the invariant glycine residue found within the SPIN β -hairpin serves the role of a hinge (26). Not only does this permit the β -hairpin to return toward the opening of the MPO active site channel, it also appears to allow for complete occlusion of access to the catalytic heme (19). Indeed, visualization of the SPIN/rhMPO complex as a molecular surface led to the attractive model that SPIN acts as a molecular plug, thereby preventing solute exchange from the MPO catalytic center (19).

Expression and Function of SPIN in Staphylococcal Evasion of Neutrophil Defense

The *S. aureus* gene that encodes SPIN (hereafter denoted *spn*) is located on genomic island α that encodes staphylococcal superantigen-like (*ssl*) proteins and other evasion proteins, such as hemolysin- α and leucocidin AB (11, 19). At the time of its initial discovery, a *spn*-like coding sequence was identified in the genome of 83 out of 84 strains in an *S. aureus* clinical isolate collection (19). The SPIN protein produced by these various *S. aureus* strains is also approximately 92% identical across all clonal lineages. This high level of conservation suggested that SPIN likely serves an important role in host colonization and pathogenesis by *S. aureus*. Subsequent serological examination of 20 different healthy humans showed substantial levels of anti-SPIN antibodies. This demonstrated that the SPIN protein is produced at some level during asymptomatic colonization or active infection of the human host (19).

As with many other staphylococcal innate immune evasion proteins, the SPIN pre-protein contains a canonical export signal that directs its secretion into the extracellular environment (19). Its target (i.e. solubilized rather than granular MPO), however, is localized within the phagosomal compartment. This suggested that expression of the *spn* gene might be upregulated in response to phagocytosis by neutrophils. qPCR studies using *S. aureus* strain USA300 cells showed an 8-fold increase in *spn* mRNA levels following phagocytosis when compared to free-living bacteria (19). Analogous experiments using a USA300 strain lacking the SaeR/S gene system that regulates production of numerous immune evasion molecules showed no significant difference in *spn* expression level between phagocytosed and non-phagocytosed bacteria (19). Examination of a USA300 reporter strain where the gene encoding green fluorescent protein (*gfp*) was placed downstream of the *spn* promoter revealed a time-dependent, statistically significant increase in the presence of GFP when phagocytosed bacteria were compared to their planktonically-grown

counterparts (19). Together, these results demonstrated that production of SPIN is regulated in response to the environment and is enhanced after entering the neutrophil phagosome.

Under normal circumstances, MPO contributes to bacterial killing within the neutrophil phagosome by catalyzing production of highly cytotoxic HOCl from hydrogen peroxide (H_2O_2) (15, 16). However, the increase in SPIN production by *S. aureus* following phagocytosis and the ability of SPIN to inhibit MPO raised questions about the contribution of SPIN to bacterial survival following phagocytosis. Given the biochemical complexities of the phagosome, a combination of *in vitro* and *ex vivo* models were used to address this issue. Replicating the conditions that lead to efficient bacterial killing in purely *in vitro* models involves many challenges. As far as MPO function is concerned, perhaps the greatest of these is ensuring a steady-state level of H_2O_2 substrate that is normally provided by the NADPH oxidase system that assembles during phagosome maturation. In this regard, fungal glucose oxidase (GO) (27) was used in lieu of NADPH oxidase to supply sublethal levels of H_2O_2 substrate for MPO in an *in vitro* assay system designed to interrogate the protective effects of SPIN on *S. aureus* killing (19). Whereas the addition of increasing levels of MPO to this assay system resulted in increasingly greater levels of killing for a laboratory strain of *S. aureus* (i.e. Newman) as well as a clinical MRSA isolate (i.e. USA300), both strains of *S. aureus* showed a significant decrease in killing when a fixed concentration of SPIN was included in the assay system (19). Furthermore, both the Newman and USA300 strains also showed a significant increase in survival compared to controls when progressively higher concentrations of SPIN were added to assays containing a fixed concentration of MPO (19). Together, these data provided proof of concept that SPIN could protect *S. aureus* from MPO-mediated killing within the neutrophil phagosome.

Although SPIN supported *S. aureus* survival in the reconstituted killing assay described above, its ability to affect a similar outcome in a more physiologically relevant scenario remained unclear. A normally favored approach for addressing such considerations would have been to compare wild-type versus *spn*-deleted strains of *S. aureus* in a laboratory animal infection model where neutrophils play a crucial role in the host response (e.g. the murine liver abscess model (28, 29)). Yet as with several other staphylococcal innate immune evasion proteins including SCIN-class complement inhibitors (30-32) and leukocidins (33-35), SPIN displayed remarkable selectivity for human MPO as a target (19). SPIN from *S. aureus* failed to bind or inhibit rodent, bovine, or equine MPO (19). Consequently, further assessments of the physiological contributions of SPIN required use of isolated human neutrophils in *ex vivo* bacterial killing assays.

MPO is generally considered a major contributor in neutrophil-mediated defense against *S. aureus*, as killing of the organism by MPO-deficient neutrophils occurs at a slower rate and is markedly less effective than seen for normal leukocytes (14, 16, 17). However, to provide additional insight into this phenomenon prior to more detailed studies, the extent to which *S. aureus* killing depends on MPO was also quantified using the membrane-permeable MPO inhibitor, AZM198 (36). Whereas 82% of *S. aureus* cells were killed within 1 hr following exposure to in-tact neutrophils and serum (to facilitate opsonophagocytosis), approximately 56% of the *S. aureus* cells were killed in an identical experiment using AZM198-treated neutrophils (19); these results suggested that MPO contributes only roughly 26% of the *S. aureus* killing activity of the phagosome under these experimental conditions. However, they must be interpreted with caution for several reasons. For example, the relative imprecision of these measurements underscores the fact that this system is intrinsically variable. Similarly, the complex nature of the protocol, the numerous components involved, and their intricate dependence on one another

suggests that this system is difficult to manipulate experimentally even under the most rigorous conditions. Finally, such experiments could not account for any confounding synergistic effects between various antimicrobial components of the phagosome that do not require MPO-derived oxidants to manifest themselves.

Given the limitations presented above and the small dynamic range of the killing assay, three different genetically-modified strains of *S. aureus* were employed to assess the role of SPIN in promoting bacterial survival within neutrophils – a wild-type Newman strain overexpressing *spn* from a plasmid (i.e. WT PlukM-*spn*), a *spn*-deleted Newman strain overexpressing *spn* from a plasmid (i.e. Δ *spn* PlukM-*spn*), and a *spn*-deleted Newman strain overexpressing *gfp* from a plasmid (i.e. Δ *spn* PlukM-*gfp*) (19). Enzyme inhibition studies showed that both *spn*-overexpressing strains produced approximately five-fold more SPIN than the parental Newman strain, while the *gfp*-overexpressing strain was devoid of MPO-inhibitory activity (19). Congruent to this observation, both strains that overexpressed *spn* showed enhanced survival at 1 hr following exposure to human neutrophils and serum when compared to the strain that lacked *spn* (19). Furthermore, comparison of the two *spn*-deleted strains that expressed either plasmid-borne *spn* or *gfp* to one another revealed a roughly 10% increase in bacterial survival in the presence of SPIN that was statistically significant ($p \leq 0.05$) (19). Although the magnitude of this effect appears mild at best and unimpressive at worst, it is impossible to ignore the fact that matured neutrophil phagosomes have been estimated to contain levels of MPO approaching 1 mM (37). It therefore seems unlikely that this enormous amount of MPO could be completely inactivated by a single immune evasion protein acting as a simple competitive inhibitor, rather than one that could instead enzymatically inactivate its target via proteolysis, ADP-ribosylation, glycosylation, etc.

Nevertheless, these data clearly show that SPIN provides a modest, but significant contribution to *S. aureus* survival within the neutrophil phagosome under the experimental conditions employed.

Defining the Structure/Function Relationships of the SPIN Protein

The initial biochemical studies on SPIN demonstrated that this novel protein acts as nanomolar-affinity, competitive inhibitor of MPO. Thereafter, determination of a SPIN/rhMPO co-crystal structure provided a useful basis for further dissection of SPINs structure/function relationships. In this regard, the lack of stabilizing structural features within the SPIN β -hairpin strongly suggested that this region might be disordered prior to binding MPO. If so, this would imply that the initial SPIN interaction with MPO must be driven by its α -helical bundle domain. To test these ideas, a combination of structural methods and deletion mutagenesis were used to great effect.

Since attempts to crystallize SPIN in the unbound state were unsuccessful, solution NMR spectroscopy was used as an alternative structural approach (38, 39). The small size (i.e. 8,380 Da) and favorable solubility properties of SPIN provided high-quality multidimensional NMR spectra that facilitated assignment of the SPIN backbone and sidechain ^1H , ^{15}N , and ^{13}C resonances (39). Aside from Phe⁴⁸ and Leu⁴⁹, all other resonances were identifiable in the ^1H - ^{15}N HSQC fingerprint spectrum of SPIN collected at 700 MHz (^1H). The TALOS-N platform was then used to calculate the secondary structure of SPIN based upon the longitudinal (R_1) and transverse (R_2) relaxation time constants for backbone resonances in the ^1H - ^{15}N HSQC spectrum, since these values are sensitive to the backbone structural rigidity of a protein. Internally rigid structures are expected to have homogenous relaxation time constants. While SPIN was characterized by a composite average R_1 value 2.49 ± 0.58 , the average R_1 of the N-terminal region was 2.90 ± 0.78 ; oppositely,

the composite average R_2 value for SPIN was 8.97 ± 1.29 but was 7.64 ± 1.33 for the N-terminal region. Similarly, the overall average R_2/R_1 was 3.72 ± 0.73 but was 2.76 ± 0.59 for the N-terminal region. Such localized deviation of these values when compared to the protein overall was indicative of backbone flexibility within the N-terminal region of SPIN. To further define the solution structure of SPIN, chemical shift index (CSI) values for $C\alpha$ and $C\beta$ SPIN resonances were calculated and compared with the BioMagResBank (BMRB) statistical database values for CSI. Residues in α -helical structures show a positive CSI for $C\alpha$ and negative for $C\beta$, while residues in β -strands show a negative CSI for $C\alpha$ and positive for $C\beta$; neutral CSI values are associated with residues in random coil like structures (40). Residues within the SPIN N-terminus exhibited significantly lower and less variable CSI values when compared to the protein as a whole (38). This confirmed the absence of regular secondary structure within the SPIN N-terminal region in the unbound state.

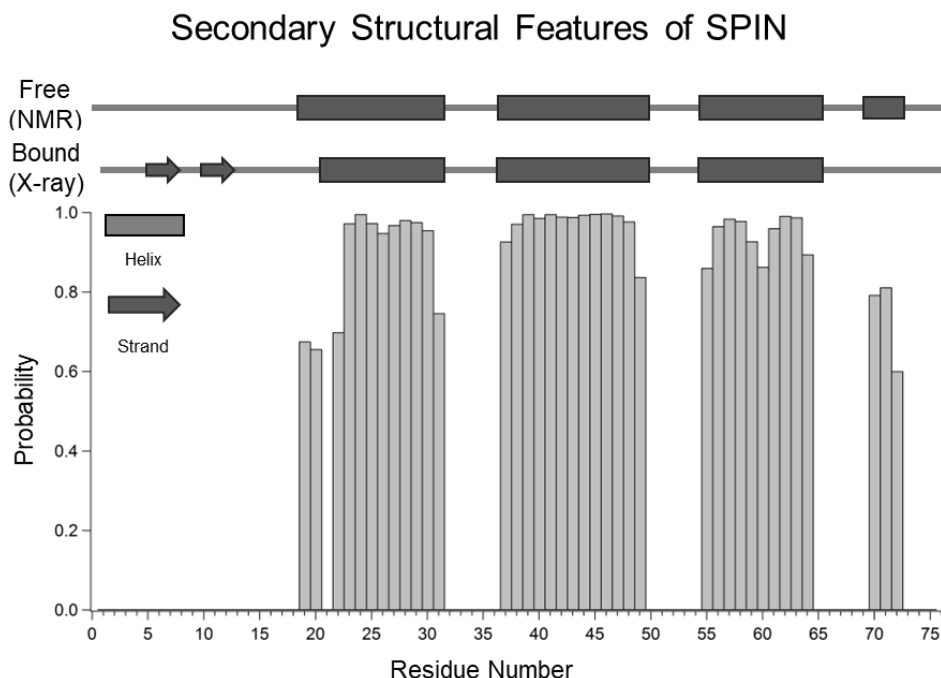


Figure 2.2 Comparison of secondary structural features of SPIN in the free and MPO-bound states.

Nuclear Magnetic Resonance (NMR) spectroscopy was used to characterize the structure of *S. aureus* SPIN in the unbound state. Following spectral assignment using standard approaches (39), the chemical shift values of resonances were used to calculate the secondary structure via the TALOS platform (41). The residue-dependent probabilities of various secondary structure elements are given on a scale from 0 to 1. Whereas the NMR data are consistent with the presence of three prominent α -helices also seen in the X-ray structure (19), the unusual β -hairpin found in the MPO-bound form of SPIN is not seen in the free state (38). These data, along with supporting biochemical studies (38), indicate that the inhibitory N-terminal β -hairpin must form following initial binding of the SPIN α -helical bundle to MPO.

Although the SPIN β -hairpin is responsible for a majority of the buried surface area in the SPIN/rhMPO complex (19), the NMR studies described above implied that the SPIN α -helical bundle predominates in terms of initial binding to MPO. To assess this experimentally, two SPIN mutants with altered N-terminal regions were developed (38). The first mutant had its N-terminus completely removed and was comprised of SPIN residues Ala⁴⁶-Lys¹⁰⁵ inclusive (hereafter SPIN⁴⁶⁻¹⁰⁵). The second mutant was used to determine how His⁴³, Asp⁴⁴, and Asp⁴⁵ impacted binding. Each of these residues was simultaneously replaced with Ala (hereafter SPIN^{43-45→AAA}), as these residues are highly conserved among SPINs (26) and form polar interactions with MPO sidechains in the SPIN/rhMPO crystal structure (19). Surface plasmon resonance studies revealed that SPIN⁴⁶⁻¹⁰⁵ and SPIN^{43-45→AAA} had approximately 3.2-fold and 3.4-fold weakened affinities for native MPO, respectively (38), that arose from enhanced dissociation rates when compared to wild-type SPIN. A subsequent crystal structure of SPIN⁴⁶⁻¹⁰⁵ bound to rhMPO determined at 2.3 Å limiting resolution and refined to $R_{\text{work}}/R_{\text{free}}$ values of 17.9% and 22.9%, respectively, showed essentially no variance from SPIN/rhMPO (38), as the C α positions from each model superimposed with an r.m.s.d. of 0.16 Å. Together, these observations reinforced the concept that the N-terminus is not essential for SPIN binding to MPO.

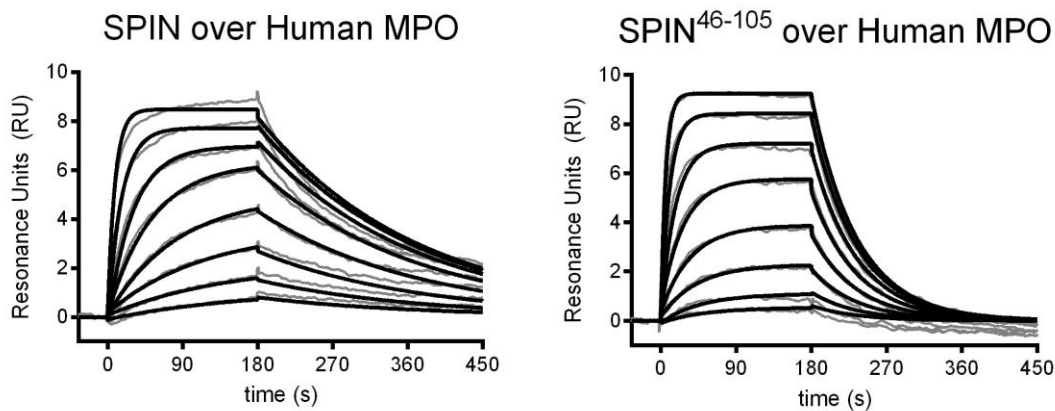


Figure 2.3 The N-terminal region of SPIN is dispensable for binding to human MPO.

Surface plasmon resonance was used to assess the interaction of soluble SPIN proteins with immobilized forms of human MPO (38). The reference-corrected sensorgrams (grey lines) obtained by injecting a two-fold concentration series ranging from 1.95 to 250 nM were analyzed by fitting to a Langmuir binding model (black lines) to derive estimates for the association rate (k_{on}), dissociation rate (k_{off}), and equilibrium dissociation constants (K_D) for each interaction. (A) Wild-type SPIN over native human MPO; $k_{on} = 4.9 \pm 0.6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{off} = 5.1 \pm 0.2 \times 10^{-3} \text{ s}^{-1}$, and $K_D = 10.5 \pm 1.8 \text{ nM}$. (B) SPIN⁴⁶⁻¹⁰⁵ over native human MPO; $k_{on} = 5.2 \pm 1.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{off} = 16.8 \pm 2.4 \times 10^{-3} \text{ s}^{-1}$, and $K_D = 32.5 \pm 3.7 \text{ nM}$. Note that the roughly 3-fold increase in the dissociation rate for this protein is responsible for the commensurate decrease in its apparent affinity for human MPO.

Further comparison of these mutants to the wild-type protein provided additional insight into the structure/function relationships of SPIN. To begin, all three proteins were examined in a functional assay where MPO-catalyzed reduction of H_2O_2 was linked to oxidation of the chromogenic substrate, o-dianisidine. Since wild-type SPIN, SPIN⁴⁶⁻¹⁰⁵, and SPIN^{43-45→AAA} all exhibited similar K_D values for MPO (38), MPO enzyme activity was measured in the presence of each SPIN protein at a single concentration sufficient to promote MPO binding (i.e. 74 nM). Although the wild-type protein inhibited MPO activity by over 90% under these conditions ($p \leq 0.0001$), neither mutant conferred any inhibition of MPO (38). These same proteins were also examined in the *in vitro* GO-MPO killing model (19), as well as a phagocytosis-dependent assay that measures bleaching of GFP-expressing *S. aureus* cells (42). Each of these assays is designed

to mimic HOCl-dependent killing within the neutrophil phagosome. Again, only wild-type SPIN showed any ability to significantly inhibit MPO in either assay. Taken together, these data established that the N-terminus of SPIN is necessary, but not sufficient for inhibiting MPO activity. In that regard it was somewhat surprising that the SPIN^{43-45→AAA} mutant had lost its capacity to inhibit MPO, since this protein bound MPO well and had an otherwise wild-type N-terminal region. This suggests that residues His⁴³, Asp⁴⁴, and Asp⁴⁵ may be critical to guiding the nascently folding SPIN N-terminus into a productive inhibitory conformation, such as that seen in the SPIN/rhMPO co-crystal structure (19, 38).

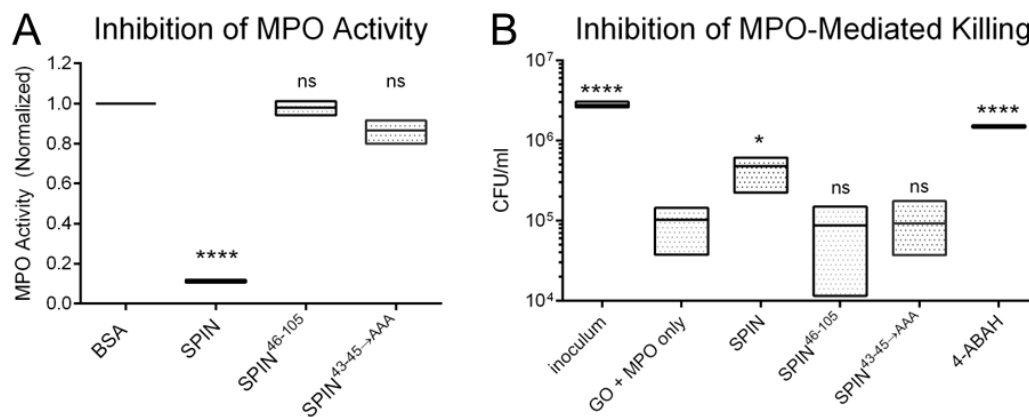


Figure 2.4 The N-terminal region of SPIN is necessary but not sufficient for inhibiting MPO.

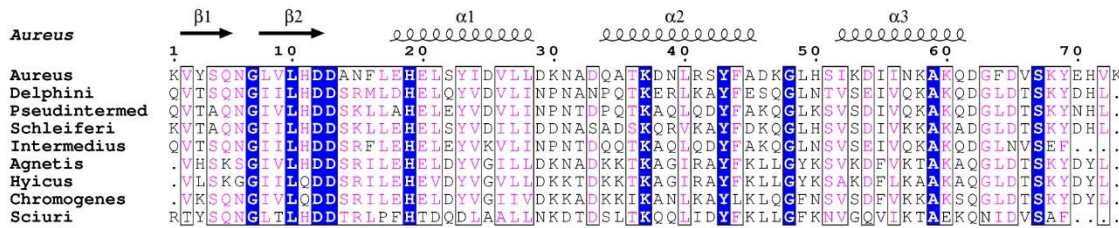
The influence of wild-type SPIN, SPIN⁴⁶⁻¹⁰⁵, and SPIN^{43-45→AAA} were compared two different assays for MPO function (38). Each experiment is represented as a box with the mean (center line) and range of the data (n=3). (A) SPIN proteins were included at a fixed concentration of 74 nM in a chromogenic assay for MPO activity. This value lies far above the K_D of each protein for MPO, so that the activity of each SPIN-bound form of MPO was measured in this experiment (38). Whereas wild-type SPIN significantly inhibited MPO activity when compared to control (BSA), neither SPIN⁴⁶⁻¹⁰⁵ nor SPIN^{43-45→AAA} had any significant effect on MPO activity. (B) SPIN proteins were also included in an assay where killing of *S. aureus* cells occurs via MPO-catalyzed generation of HOCl (19). Only wild-type SPIN significantly inhibited MPO in this system, as judged by the nearly 10-fold increase in survival of *S. aureus* cells when compared to GO-MPO alone. The small molecule, 4-aminobenzoic acid hydrazide (4-ABAH), was included as positive control for inhibition of MPO. Note that the SPIN^{43-45→AAA} mutant behaved similarly to the SPIN⁴⁶⁻¹⁰⁵ mutant in each assay. This demonstrated that simply having a full-length N-terminus is not enough to drive inhibition of MPO by SPIN.

Identification and Characterization of SPIN Homologs from Other Staphylococci

Whereas the *spn* gene was found to be highly conserved across human- and animal-derived *S. aureus* strains (19), the distribution of homologous *spn* genes throughout more distantly related species of staphylococci was unclear. To address this question, the SPIN protein sequence from *S. aureus* strain Newman was used to query the NCBI non-redundant protein database for any uncharacterized molecules with sequence identity great enough to suggest they might also function as MPO inhibitors. Eight proteins from various staphylococcal species pathogenic to humans, livestock, and/or domestic companion animals were identified from this search (26). The putative secretion signal peptide cleavage site was determined for each protein using SignalP (43). Thereafter, multiple sequence alignment of the matured sequences revealed that this cohort of uncharacterized proteins had amino acid identities with one another that ranged from a low of 29% to a high of 91% (26).

Phylogenetic analyses revealed multiple relationships among the sequences of these SPIN homologs (26). For example, the SPIN proteins from *S. hyicus* and *S. agnetis* show approximately 85% identity with one another. This aligns with a previous report which proposed an evolutionary relationship between these two species (44). Oppositely, SPIN from *S. sciuri* shows comparatively weaker conservation with other SPIN sequences at only 29 to 39% identity. Again, this observation is in agreement with previous work which suggested that *S. sciuri* represents a more distant evolutionary branch of these otherwise closely related bacteria (45, 46). Finally, the SPIN proteins from *S. shleiferi* and members of the *Staphylococcus intermedius* group (SIG), which includes *S. intermedius*, *S. pseudintermedius*, and *S. delphini*, share approximately 59% sequence identity with one another. These SPIN proteins are also noteworthy since they share the highest overall sequence identities with SPIN-*aureus*, ranging from 53% to 57%.

A Sequence Features of SPIN Homologs



B

Human MPO Inhibition by SPIN Homologs

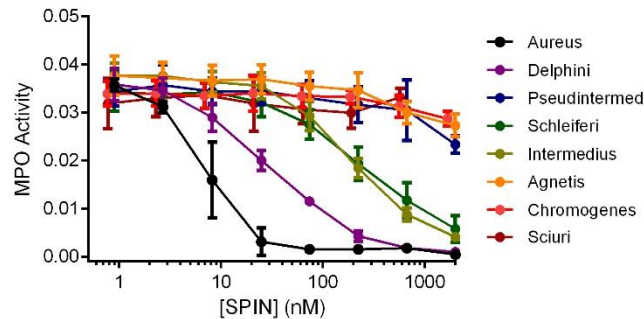


Figure 2.5 Sequence features of a panel of SPIN homologs and their inhibition of human MPO.

(A) BLAST searching in the NCBI non-redundant protein database was used to identify uncharacterized proteins with homology to *S. aureus* SPIN (26). The predicted matured forms of all proteins were aligned using Clustal Omega and are presented beneath the secondary structure elements of *S. aureus* SPIN in the MPO-bound form (19). Invariant residues are shown as white letters inside blue fields, while residues found in a majority these sequences appear in magenta letters. Only 10 of the 73 residues comprise the matured form of *S. aureus* SPIN are invariant among these sequences. (B) Recombinant forms of various SPIN homologs were included in an assay for human MPO activity over a broad range of concentrations (26). In addition to *S. aureus* SPIN, the homologs from *S. delphini*, *S. schleiferi*, and *S. intermedius* exhibited dose-dependent inhibition of human MPO. The remaining homologs failed to inhibit human MPO at the highest concentrations tested. This observation is in accordance with prior results which suggest that the inhibitory activities of the SPIN protein are host restricted (19). The y-axis represents the initial velocity of the chromogenic reaction.

Despite their significant levels of sequence identity to *S. aureus* SPIN, it remained to be determined if any of these SPIN-like proteins could also bind MPO and inhibit its enzymatic activity. To that end, a synthetic coding sequence for the matured form of each SPIN homolog was produced and used to express and purify a recombinant form of the protein (26). Surface plasmon resonance (SPR) was then used to examine the ability of each SPIN homolog to bind native human MPO that had been immobilized on the biosensor surface. Initial experiments revealed that several

of these proteins exhibited dose-depending binding to native human MPO (as will be described below), but that SPINs from *S. chromogenes*, *S. pseudintermedius*, and *S. sciuri* failed to do so. These three proteins, along with SPIN from *S. hyicus* which was unstable and prone to degradation, were therefore excluded from further interaction analysis (26).

Full kinetic profiling of the interactions between immobilized human MPO and the SPINs from *S. delphini*, *S. intermedius*, *S. schleiferi*, and *S. agnetis* was carried out alongside *S. aureus* SPIN as a comparator. This allowed for determination of the association (k_a) and dissociation (k_d) rate constants, as well as the apparent equilibrium dissociation constant (K_D) for each interaction pair. While the K_D of 15.9 ± 2.1 nM for SPIN-*aureus* was in agreement with previous observations (19, 38), the affinities of the other interactions were much more variable. Significantly, none of the homologs bound human MPO as strongly as SPIN from *S. aureus* (26). SPIN from *S. agnetis* displayed the next highest affinity with a K_D of 41.8 ± 1.1 nM, followed by the homologs from *S. schleiferi*, *S. delphini*, and *S. intermedius* at 230 ± 42.1 nM, 310 ± 41.5 nM, and 984 ± 274 nM, respectively. These values corresponded to decreases in affinity for human MPO that ranged from 2.6- to 61.9-fold when compared to the *S. aureus* protein. Although the dissociation rate constants for the SPIN homologs were consistent as a group and ranged from $2.36 \pm 0.10 \times 10^{-3} \text{ s}^{-1}$ to $3.34 \pm 0.17 \times 10^{-3} \text{ s}^{-1}$, the association rates constants were more variable. These values ranged from $20.10 \pm 2.22 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for SPIN-*aureus* to $0.35 \pm 0.08 \times 10^{-4} \text{ M}^{-1}\text{s}^{-1}$ for SPIN-*intermedius*. Consequently, the differences in affinity for human MPO among this cohort of SPIN homologs can be attributed primarily to variations in their respective association rate constants.

Previous studies on mutants of *S. aureus* SPIN showed that binding to MPO is necessary but not sufficient for inhibiting MPO enzymatic activity (38). Select members from this panel of SPIN homologs were therefore examined in a colorimetric assay for MPO activity (26). In line

with previous observations (19, 38), SPIN from *S. aureus* inhibited the peroxidase activity of human MPO with an IC_{50} value very near its K_D for MPO (i.e. ~ 10 nM). Each of the homologs that failed to bind human MPO (i.e. SPIN-*sciuri*, SPIN-*chromogenes*, and SPIN-*pseudintermedius*) likewise failed to inhibit MPO activity. However, three SPIN variants, including those from *S. delphini* ($IC_{50} \sim 29.7$ nM), *S. intermedius* ($IC_{50} \sim 222$ nM), and *S. schleiferi* ($IC_{50} \sim 261$ nM), inhibited the peroxidase activity of human MPO in a dose-dependent manner. Although the reason underlying the discrepancy between the observed equilibrium dissociation constant and half-inhibitory concentration for these SPIN homologs remains unclear, each of these proteins shows an ability to both bind and inhibit human MPO. The same cannot be said for the *S. agnetis* homolog, which did not inhibit human MPO even though it binds with a robust affinity ($K_D \sim 41.8$ nM). Indeed, the properties of SPIN from *S. agnetis* resembled the site-directed mutant, SPIN^{43-45→AAA}, which also failed to inhibit MPO despite a near wild-type affinity (38). Despite these differences in affinity for and potency against human MPO, a subset of these homologs clearly maintained the inhibitory activity first reported for the *S. aureus* SPIN protein. This strongly suggests that the ability to inhibit MPO activity is broadly distributed among this branch of staphylococci, and not restricted to organisms like *S. aureus* which exhibit substantial preference for the human host.

Structure of SPIN from *S. delphini* Bound to Recombinant Human MPO Provides a Basis for Comparative Studies on SPIN-class MPO Inhibitors

Considering the entire cohort of recently identified homologs, SPIN from *S. delphini* inhibited human MPO with an IC_{50} value second only to *S. aureus* SPIN. This suggested that further study of SPIN from *S. delphini* could not only define the conserved physical properties that

manifest as inhibition of MPO, but also help to identify features that lead to differences in structure/activity profiles among SPINs. To that end, SPIN from *S. delphini* was crystallized with rhMPO, and the structure was solved at 2.4 Å limiting resolution and refined to $R_{\text{work}}/R_{\text{free}}$ values of 18.5% and 21.8%, respectively (26). Unlike the structure of *S. aureus* SPIN bound to rhMPO (19), the structure of this complex contained a covalently bound heme modeled at full occupancy within the rhMPO active site; this observation ruled out the possibility that binding of SPIN-class inhibitors to MPO might somehow promote loss of heme from the MPO active site, and thereby inhibit MPO activity by displacing the essential heme cofactor. That difference aside, further comparison of the *S. delphini* SPIN/rhMPO complex with that of *S. aureus* SPIN/rhMPO revealed extensive similarities between the two structures. Not only did 539 of the corresponding C α positions within each complex superimpose with an r.m.s.d. of 0.289 Å, 61 C α positions from the two different SPIN molecules superimposed with an r.m.s.d. of 0.812 Å. In this regard, the only noteworthy regions of tertiary structural divergence between the *S. delphini* and *S. aureus* SPIN proteins were found in the extreme N- and C-termini of the respective polypeptides.

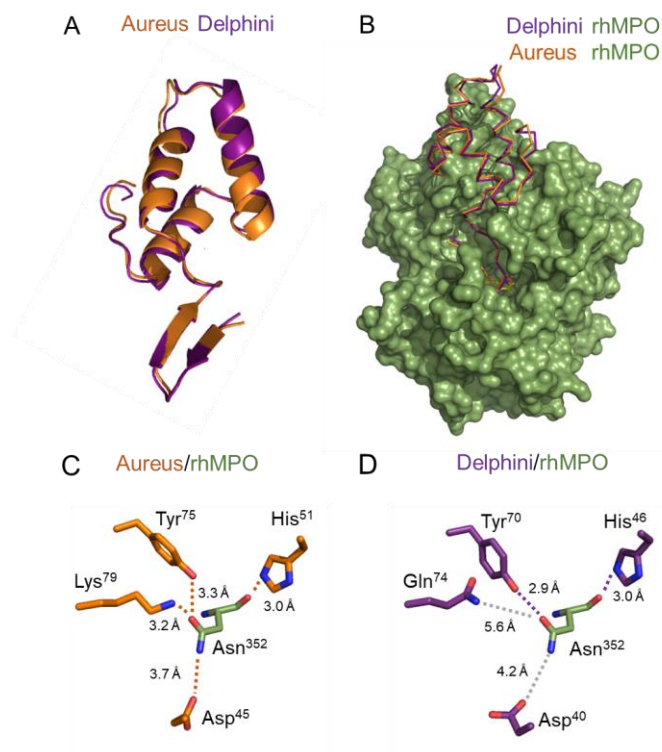


Figure 2.6 Structural characterization of SPIN from *S. delphini* bound to a recombinant form of human MPO.

SPIN from *S. delphini* was crystallized bound to rhMPO and the structure was solved to 2.4 Å limiting resolution (26). (A) Superposition of the SPIN proteins from *S. aureus* (orange) and *S. delphini* (purple), drawn as ribbon diagrams. The inhibitory N-terminal β -hairpin appears at the bottom of the image. (B) Superposition of the crystal structure of SPIN from *S. delphini* bound to rhMPO (PDB code 6BMT (26)) with that of *S. aureus* SPIN bound to rhMPO (PDB code 5UZU (19)). The SPIN proteins are drawn as $\text{C}\alpha$ wires using the same coloring scheme as panel A. This illustrates the conserved positing of each SPIN inhibitor relative to rhMPO (olive surface). (C) *S. aureus* SPIN residues (carbon atoms colored orange) and the contacts formed with Asn³⁵² of rhMPO (carbon atoms colored olive). Likely polar interactions, along with their distances, are shown as orange dashed lines. (D) *S. delphini* SPIN residues (carbon atoms colored purple) and the contacts formed with Asn³⁵² of rhMPO. Likely polar interactions and distances are shown as purple dashed lines, while interactions that fail to form due to excessive distance are shown as grey dashed lines. These observations suggest that the decreased affinity of *S. delphini* SPIN for human MPO arises partly from local structural changes when compared to the *S. aureus* SPIN protein (26).

The SPIN protein from *S. delphini* shares 53% amino acid identity to its *S. aureus* counterpart but binds comparatively weakly to and is a less potent inhibitor of human MPO (26). Consequently, the *S. delphini* SPIN/rhMPO complex was analyzed for any sequence-dependent

differences in local structure features that could explain the partial impairment of its function (26). The entire contact interface between *S. delphini* SPIN and rhMPO displayed a similar level of shape complementarity to the *S. aureus* SPIN complex, with s.c. coefficients of 0.715 and 0.704, respectively (47). Restricting this analysis to the helical-bundle domains alone, however, revealed a far greater level of shape complementarity for the *S. aureus* complex, with recalculated s.c. coefficients of 0.715 and 0.774, respectively. The difference in shape complementarity between the two helical-bundle domains resulted in a lower overall buried surface area of 1,365 Å² for the *S. delphini* protein when compared to 1,633 Å² for *S. aureus* SPIN (19). As shape complementarity and buried surface area provide reasonable benchmarks for the hydrophobic interactions that are a major determinant in driving binding affinities, the observations presented above suggest that the *S. aureus* SPIN protein is simply a better fit for human MPO than is *S. delphini* SPIN.

The sequence differences between the *S. delphini* and *S. aureus* SPIN proteins also changed the polar interactions they formed with rhMPO (26). Whereas 14 residues from *S. aureus* SPIN formed one or more polar interactions with groups found on rhMPO, only 11 residues from *S. delphini* SPIN did the same. Further inspection showed that 7 of these 11 residues were conserved between these two SPINs and formed essentially identical interactions with rhMPO. Another conserved residue, Asp⁴⁵ in *S. aureus* and Asp⁴⁰ in *S. delphini*, was positioned too far in the structure of *S. delphini* SPIN bound to rhMPO to interact with the Asn³⁵² sidechain derived from the enzyme (i.e. 3.7 Å versus 4.2 Å, respectively). Three other residues from *S. delphini* found at the interface with rhMPO (i.e. Gln²⁸, Thr³⁰, and Gln⁷⁴) constitute significant changes in sidechain properties with their corresponding positions in *S. aureus* SPIN (i.e. Lys³³, Tyr³⁵, and Lys⁷⁹), and thus fail to form equivalent polar interactions with rhMPO. Of these, only Gln⁷⁴ from *S. delphini* is part of the α-helical bundle domain that drives the initial interaction between SPIN and MPO

(38). Interestingly, the change from Lys⁷⁹ in *S. aureus* to Gln⁷⁴ in *S. delphini* prevents formation of a hydrogen bond with the Asn³⁵² sidechain of rhMPO. This situation is reminiscent of the relationship between *S. aureus* Asp⁴⁵ and *S. delphini* Asp⁴⁰ described earlier in this paragraph, which also target the same Asn³⁵² sidechain of rhMPO (26). Thus, the quantitative differences in function between the *S. aureus* and *S. delphini* SPIN proteins appear to arise from sequence-dependent changes in local structural features that influence both surface complementarity and the orientation of key functional groups at the inhibitor interface with human MPO.

Conclusion

Specific small-molecule inhibitors of MPO have been known for a decade and perhaps longer (36, 48, 49), yet the only proteinaceous inhibitor of MPO described prior to the identification of SPIN was plasma-resident ceruloplasmin (50-52). The discovery of SPIN therefore provided the first evidence that inhibition of MPO activity by a pathogen-derived molecule could be advantageous in helping diminish a critical defense mechanism of the host (12, 19). While studies on SPIN as well as other staphylococcal innate immune evasion proteins that act within neutrophil phagosome have expanded understanding of the molecular basis of pathogenesis, SPIN also provides a unique tool for dissecting the structure/function relationships of a protein whose only known activity is inhibition of MPO *in situ*. Though much work remains to be done, comparative studies on *S. aureus* SPIN and its homologs have already identified conserved features that provide for high-affinity binding and inhibition of MPO. They have also provided a biologically relevant basis for defining quantitative structure/activity relationships of MPO inhibitors and a powerful means for defining the physical basis behind the host-selectivity of virulence proteins. In many ways, the story of this novel MPO inhibitor is only beginning to be told, with many important insights certain to follow throughout the years ahead.

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Chapter 3 - Staphylococcal Peroxidase Inhibitor (SPIN): Investigation of inhibitory N-terminal domain via a stabilizing N-terminus insertion

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Abstract (248 words)

Staphylococcus aureus secretes an array of small proteins that inhibit key enzyme-catalyzed reactions necessary for proper function of the human innate immune system. Among these, the Staphylococcal Peroxidase Inhibitor, SPIN, blocks the activity of myeloperoxidase (MPO) and thereby disrupts the HOCl-generating system of neutrophils. Previous studies on *S. aureus* SPIN have shown that it relies on a C-terminal α -helical bundle domain to mediate initial binding to MPO, but requires a disordered N-terminal region to fold into a β -hairpin conformation to inhibit MPO activity. To further investigate the structure/function relationship of SPIN, we introduced two cysteine residues into its N-terminal region to trap SPIN in its MPO-bound conformation and characterized the modified protein, which we refer to here as SPIN-CYS. Although control experiments confirmed the presence of the disulfide bond in SPIN-CYS, solution structure determination revealed that the N-terminal region of SPIN-CYS adopted a physically constrained series of lariat-like structures rather than a well-defined β -hairpin. Nevertheless, SPIN-CYS exhibited a gain in inhibitory potency against human MPO when compared to wild-type SPIN. This gain of function persisted even in the presence of deleterious mutations within the C-terminal α -helical bundle domain. Surface plasmon resonance studies showed that the gain in potency arose through an increase in apparent affinity of SPIN-CYS for MPO, which was driven primarily by an increased association rate with MPO when compared to wild-type SPIN. Together, this work provides new information on the coupled binding and folding events required to manifest biological activity of this unusual MPO inhibitor.

KEYWORDS

Myeloperoxidase, Inhibitor, Structure/Function, *Staphylococcus aureus*, Immune Evasion

Introduction

Neutrophils are the most abundant leukocytes in humans and are critical components of the innate immune response against bacterial pathogens (Reviewed in (1, 2)). Following opsonization of bacteria by either complement fragments or antibodies, neutrophils engulf the bacteria into their phagosomal compartment and thereafter mobilize their granule-resident defenses to affect intracellular killing. Although neutrophils possess a diverse portfolio of antibacterial molecules including proteases, lysozymes, and antimicrobial peptides (1, 2), they are perhaps best known for their oxidative killing system wherein the heme-containing enzyme, myeloperoxidase (MPO), plays an essential role (3, 4). MPO is the most abundant protein in neutrophils and is present at unusually high levels within the phagosome, where it catalyzes the rapid generation of cytotoxic levels of HOCl and other hypohalous acids (5). However, this potent killing mechanism is thought to have exerted strong selective pressure on microorganisms to evolve strategies for escaping its action. Indeed, the Gram-positive bacterium *Staphylococcus aureus* and several closely related staphylococcal species secrete a small (~8.4 kDa), but potent MPO inhibitor known as SPIN (for Staphylococcal Peroxidase INhibitor) (6, 7). Studies on *S. aureus* showed that expression of the gene encoding SPIN is induced upon exposure to oxidant and following bacterial phagocytosis, strongly suggesting that SPIN functions primarily within the phagosome (6). Furthermore, cells of a *S. aureus* strain deficient in SPIN are also more readily killed by isolated human neutrophils than are cells of an isogenic control strain (6). Together, these data suggest that SPIN contributes to *S. aureus* survival within the human neutrophil phagosome by inhibiting HOCl generation via MPO.

SPIN from *Staphylococcus aureus* shares no significant sequence identity with any other proteins (6, 8), aside from the orthologs from other closely related bacteria mentioned above (7). Therefore, extensive structure/function studies were required to understand its apparently unique

mode of action as an MPO inhibitor (Reviewed in (9)). Despite its small size of only ~73 residues, the initial X-ray crystal structure of SPIN bound to a recombinant form of human MPO strongly suggested that SPIN consists of two functional domains: an MPO-binding/targeting α -helical bundle domain of ~60-residues at its C-terminus, and an inhibitory ~13-residue β -hairpin that lies at its N-terminus (6). The N-terminal β -hairpin presented a paradox, though, as it lacks obvious stabilizing structural features such as disulfide bonds; consistent with this, NMR studies on the full-length SPIN protein in the absence of MPO revealed that this N-terminal region is in fact disordered prior to MPO binding (8, 10). Subsequent characterization of a SPIN mutant lacking the N-terminal region showed that this protein retains near wild-type binding affinity, but is completely devoid of the ability to inhibit MPO (8). This led us to propose an ordered “bind, fold, then inhibit” mechanism for inhibition of MPO by SPIN (8, 9).

Visualizing the structure of SPIN bound to recombinant human MPO as a molecular surface has been particularly informative in suggesting a physical basis for SPIN function (6, 9). In such representations, the SPIN N-terminus appears to serve as a molecular plug that occludes free solute exchange through the enzyme active site channel leading to the catalytic heme (6). However, as pointed out by Leitgeb and coworkers (11), many biologically-relevant substrates of MPO, including the initial oxidant, H_2O_2 , as well as halide anions, notably Cl^- and Br^- , are roughly the same size as H_2O (11). This suggested that it might still be possible for solutes like these to enter the MPO active site. Using an array of complementary biophysical and kinetic methods, Leitgeb et al. showed that SPIN does not fully block halide oxidation by MPO (11). Therefore, their observations showed that the molecular plug model is not entirely accurate. Nevertheless, the same authors also found that SPIN becomes an increasingly effective inhibitor as the size of the substrate to be oxidized increases (11). Collectively the data presented by Leitgeb et al. seemed to

suggest that the inhibitory N-terminal β -hairpin of SPIN fits rather loosely inside the MPO active site channel even though it occupies much of the volume available. Whether this “loose fitting” inhibition goes so far as to involve a complete unfolding of the SPIN β -hairpin while the α -helical bundle domain remains associated with MPO has yet to be addressed through experimental methods. For that matter, little is known of how the SPIN N-terminus folds and inserts into the MPO active site channel following initial binding to the enzyme.

Zhang et al. recently described a series of atomistic molecular dynamics simulations investigating the coupled binding and folding mechanism of the SPIN N-terminus (12). These authors concluded that binding and folding of the *S. aureus* SPIN N-terminus is a highly cooperative process. Considering their observations, we wondered if restricting the structural landscape accessible to the SPIN N-terminus might result in functional consequences. It is well-documented that the *S. aureus* SPIN N-terminus lacks biochemical features that lead to intrinsic stabilization of the β -hairpin seen in its MPO-bound form (6, 8, 9). However, we were able to leverage existing structural information to generate a mutant of *S. aureus* SPIN that contains an intramolecular disulfide bond within its N-terminus. In this report, we characterize the structure of this disulfide-insertion mutant, which we refer to as SPIN-CYS. We show that this disulfide results in a mild gain-of-function in MPO inhibitory potency that is driven by an increase in its apparent affinity for MPO, primarily by increasing the association rate of the inhibitor for MPO. We also find that gain-of-function caused by this disulfide insertion remains even when other residues critical for mediating initial MPO binding have been removed by site-directed mutagenesis (13). Together, this work provides new insights into how the structural features of the SPIN N-terminus influence its function as the essential determinant of MPO inhibition in this unusual staphylococcal innate immune evasion protein.

Experimental

2.1. Proteins

Native myeloperoxidase isolated from purulent human sputum was obtained from Elastin Products Corp., while enzyme isolated from quiescent human neutrophils was obtained from Planta Natural Products. Both enzymes were reconstituted in 50 mM sodium phosphate (pH 7.0) and quantitated by measuring the heme absorbance at 430 nm (ϵ : 91 mM⁻¹cm⁻¹). Following reconstitution, the enzymes were stored until further use per the manufacturer's recommendations.

All SPIN proteins were obtained in recombinant form according to the general methods described previously (8). Gene fragments encoding the matured form of *S. aureus* SPIN, as well as various truncation and site-directed mutants, were subcloned into the bacterial expression vector pT7HMT (14) and obtained from GenScript. Following overexpression in *E. coli* strain BL21(DE3) cells, the recombinant SPIN protein was isolated from a clarified cell extract using immobilized metal ion affinity chromatography. The target recombinant protein was cleaved from the affinity tag by digestion with TEV protease. Thereafter, it was separated from the TEV protease and residual digestion products by a second round of immobilized metal ion affinity chromatography wherein the unbound fraction was collected and processed further. Final purification was achieved by size-exclusion chromatography using a Superdex 30 26/60 column attached to an AKTA format FPLC (Cytiva Life Sciences). Following confirmation of purity by SDS-PAGE, each protein was buffer exchanged into ddH₂O, separated into aliquots, and lyophilized for long-term storage.

Isotopically enriched forms of SPIN-CYS for NMR studies were expressed in *E. coli* strain BL21(DE3) cells using minimal media enriched with either ¹⁵NH₄Cl and/or [¹³C]-glucose

according to the general methods described in an earlier publication (15). These forms of SPIN were overexpressed, purified, and processed for long-term storage as described above.

2.2. Solution NMR spectroscopy

Isotopically ^{15}N - and/or ^{13}C -labeled forms of SPIN-CYS were dissolved at concentrations of ~ 1 mM in a buffer containing 50 mM sodium phosphate (pH 6.5), 50 mM sodium chloride, with 5% (v/v) D_2O as a lock solvent and placed in a 5 mm NMR tubes. All NMR measurements were conducted at a temperature of 25 °C using a 600 MHz Bruker Avance Neo NMR instrument equipped with 5 mm HCN triple resonance cryogenically cooled probe. To facilitate the assignment of both backbone and sidechain resonances, various multidimensional spectra were collected. ^{15}N -HSQC, Dipsi-TOCSY, CCCONH, and H(CCCO)NH spectra were employed for identifying spin systems, while HNCA, HNCACB, HN(CA)CO, HNCO, and HN(CO)CA spectra were utilized for the sequential assignment of SPIN-CYS. Additionally, ^{15}N -edited NOESY spectra were recorded with mixing times of 60, 100, and 150 ms to allow for a semi-quantitative calculation of distances between nuclei. All triple resonance NMR experiments were acquired using non-uniform sampling. Uniformly sampled data were obtained using reconstruction methods such as hmsIST, SMILE, NMRPipe IST, and RIST as implemented in the NMRPipe software (16).

Peak picking and resonance assignments were carried out using CARA NMR software. These assignments were then used to predict dihedral angles and secondary structure using TALOS-N (17). Structure calculation was performed using Cyana v2.1 (18) with hydrogen-bonding constraints introduced during the final structure calculation. These constraints were obtained experimentally from ^1H - ^{15}N HSQC spectra collected over a 24-hour period on a sample dissolved in 100% (v/v) D_2O . 20 of the 300 lowest energy structures were selected for preparation of the final structural ensemble. Cyana v2.1 (18), PyMOL, PROCHECK_NMR (19), and the

Protein Structure Validation Software Suite (PSVS) were used to assess structure quality. Chemical shifts, structural restraints (distance, dihedral, and H-bonding restraints), and atomic coordinates have been deposited in the BMRB (entry no. 52103) and RCSB (PDB ID 9BD0).

2.3. Steady-State Kinetic Measurement of Myeloperoxidase Inhibition by SPIN Proteins

A coupled colorimetric assay consisting of H₂O₂, vanillic acid (VA), and 4-aminoantipyrine (AAP) was used to measure MPO activity within the peroxidase cycle (20). In this reaction, oxidized AAP condenses with VA to form a quinoneimine dye that is followed spectrophotometrically at 510 nm (ϵ : 6.98 mM⁻¹cm⁻¹) (21). To quantify MPO activity, 170 μ L of a chromogenic assay mixture consisting of 4.24 nM MPO (enzyme from Planta Natural Products), 7 mM VA and 5mM AAP were added to 170 μ L of 50 mM sodium phosphate buffer (pH 7.0) containing H₂O₂ concentrations ranging from $\sim$ 5 μ M to $\sim$ 1.1 mM; the formation of the quinoneimine product was then followed spectrophotometrically in a 96-well plate format. Triplicate data (n = 3) were fit to a Michaelis-Menten equation with an additional substrate inhibition component (uncompetitive inhibition) to determine the k_{cat} , K_m and K_I with respect to H₂O₂.

$$y = \frac{k_{cat} * [S]}{K_m + ([S] * [1 + \frac{[S]}{K_I}])}$$

To investigate inhibition of MPO by SPIN proteins, an H₂O₂-free reaction mixture analogous to that described above was prepared and incubated with varying concentrations of SPIN proteins for 1.5 h at room temperature. Complete reactions were prepared in a 96-well plate by rapidly mixing 170 μ L of each sample above with an equal volume of $\sim$ 112 μ M H₂O₂ in 50 mM sodium phosphate buffer (pH 7.0); the absorbance was then followed continuously every 25

seconds for 30 min at 25 °C using a Versa_{max} tunable microplate reader. Each inhibition assay was performed in at least three sets of three replicates and the IC₅₀ values were estimated by fitting the data to an IC₅₀ equation containing four variables (i.e. y_{max} (fixed to the average value of uninhibited rates), y_{min}, logIC₅₀ and hillslope (allowed to float)) using GraphPad Prism.

$$y = \frac{y_{\min} + (y_{\max} - y_{\min})}{(1 + 10^{((\log IC_{50} - x) * \text{hillslope}))}}$$

2.4. Surface Plasmon Resonance

Protein-protein interactions between the SPIN mutants and MPO were investigated by Surface Plasmon Resonance using a Biacore T-200 instrument (Cytiva Life Sciences) and the general methods described earlier (6–8). All experiments were performed at 25 °C using HBS-T as a running buffer and a flow rate of 30 µL/min. Replicate biosensors were created on three separate experimental flow cells of a CMD-200M chip (Xantec Bioanalytics GmbH) using standard NHS/EDC chemistry to couple human MPO (20 µg/mL in 10 mM sodium acetate (pH 5.5)) to the surface. Two different sensor chips were used in this study. MPO obtained from Elastin Products Corp was used to assess binding of SPIN-CYS, SPIN-WT, and SPIN-dN; the final ligand densities on each flow cell were 1041.7, 758.5, and 643.8 resonance units (RU), respectively. MPO obtained from Planta Natural Products was used to assess binding of all other SPIN variants; the final ligand densities on each flow cell were 855.1, 798.4, and 701 RU, respectively. One flow cell on each chip was activated by NHS/EDC and immediately quenched by ethanolamine to serve as the reference surface. Two-fold serial dilutions (1.93-1,000 nM) of each SPIN protein were injected over the flow cells with a contact time of 3 min, followed by a 5 min dissociation phase. For the L49A-CYS and E52A-CYS mutants (1.93-2,000 nM), as well as the Y55A-CYS mutant (3-50,000 nM), higher concentration ranges were used to obtain a more accurate estimation of the

apparent rate constants. A 0.5 min pulse of 0.1 M glycine (pH 10.0) containing 1 M NaCl was applied to regenerate the surface between injection cycles. Kinetic evaluation was performed on each reference-subtracted injection series using Biacore T-200 evaluation software (Cytiva). A two-state binding model was used to determine the apparent rate constants (i.e. k_{a1} , k_{d1} , k_{a2} , and k_{d2}) and equilibrium constant (i.e. K_D) from the resulting fits.

2.5. Miscellaneous

Representations of secondary structure as a function of amino acid sequence were generated using EsPript (22). Images of protein structures were rendered using PyMol (<https://pymol.org/>). Analyses of protein-protein interfaces were carried out using the EBI-PISA server (<https://www.ebi.ac.uk/pdbe/pisa/>) (23).

Results

3.1. Rationale for and Structural Characterization of a Disulfide-Insertion Mutant in S. aureus SPIN

The “bind, fold, then inhibit” mechanism for SPIN action is an ordered process (8, 9): the C-terminal α -helical bundle domain is responsible for initial MPO binding while the previously disordered N-terminal region folds into the β -hairpin structure required to inhibit MPO catalytic activity. The N-terminal region of SPIN is comprised of only 13 residues and examination of its structure in the MPO-bound state reveals no obvious features to stabilize the β -hairpin in the absence of MPO (6, 8). Interestingly, the sidechains of positions V34 and D44 lie at opposing ends of the SPIN β -hairpin yet are only ~ 3.6 Å distance from one another in the MPO-bound structure (6). Therefore, we hypothesized that inserting a pair of cysteine residues at positions V34 and D44 would create a disulfide bond, stabilize the SPIN β -hairpin in its MPO-bound conformation, and thereby promote inhibitory activity (Fig. 3.1A).

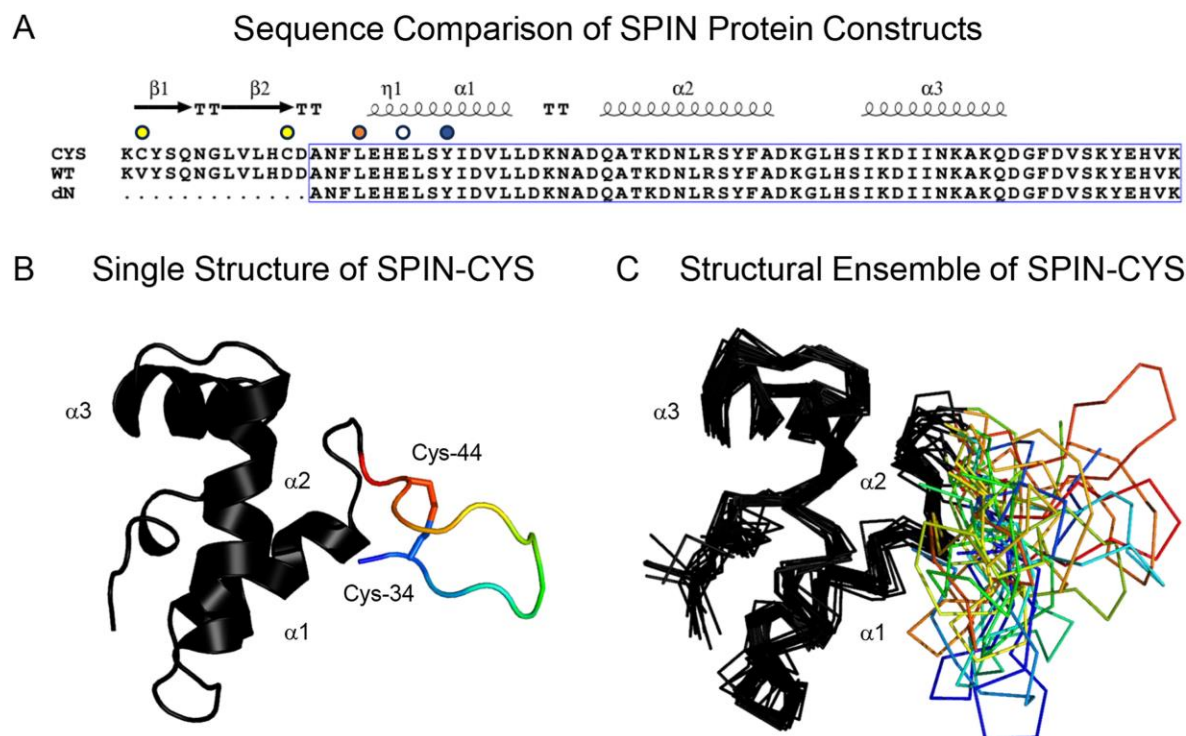


Figure 3.1 Sequence of the Staphylococcal Complement Inhibitor, SPIN, and Solution Structure of the Disulfide-Insertion Mutant, SPIN-CYS.

(A) Sequence alignment of *S. aureus* SPIN proteins investigated here, as rendered by EsPript (22). Secondary structure elements of the MPO-bound form of *S. aureus* SPIN (6) are shown above the sequence alignment, where the residues corresponding to the α -helical bundle domain are enclosed in a blue rectangle. Residues V34 and D44, which were replaced by cysteines in SPIN-CYS, are illustrated with yellow circles. The locations of residues utilized in mutagenesis studies are indicated: L49, orange circles; E52, clear circles; and Y55, blue circles. (B) Structure of a representative solution from the NMR structure calculation for SPIN-CYS. The SPIN polypeptide is depicted as a black ribbon diagram. Residues comprising the N-terminal region are colored from C34 (blue) to D45 (red), with the inserted disulfide shown in ball and stick convention. Supporting data pertaining to structure solution and calculation are shown in Table 3.1. (C) Ensemble of the 20 lowest-energy structure solutions for SPIN-CYS, as determined by NMR. The color scheme is identical to that used in Panel B, except that the N-termini for individual solutions are depicted with single colors. Note that only $C\alpha$ positions are shown for purposes of clarity.

To investigate this possibility, we overexpressed and purified a V34C/D44C double mutant in *S. aureus* SPIN (hereafter SPIN-CYS). Characterization of the recombinant protein using an Ellman assay revealed no free sulfhydryls in the sample (*Data Not Shown*); since SPIN has no other naturally occurring cysteine residues, and because the protein behaved as a monomer in solution, this result suggested that the sidechains of C34 and C44 had indeed oxidized to form the

intended disulfide bond. Thereafter, we expressed and purified $^{15}\text{N}/^{13}\text{C}$ isotopically labeled SPIN-CYS and collected its 3D HNCACB spectrum; this experiment showed $\text{C}\beta$ resonances at 43.6 and 43.5 ppm for C34 and C44, respectively, which matched well the expected value of 43 ppm (^{13}C) if the residue were in a disulfide bond (Fig. 3S1) (24). We then used heteronuclear NMR methods to solve the solution structure of SPIN-CYS (Fig. 3.1B, 3.1C, & Table 3.1). Examination of a representative solution from the ensemble of the 20 lowest-energy structures showed that the C-terminal helical bundle domain of SPIN is not affected by the two mutations, and that the sidechains of C34 and C44 do indeed form a disulfide bond consistent with the observations described above. Although the N-terminal region of SPIN-CYS adopts a more constrained conformation resembling a β -hairpin in certain solutions (Fig. 3.1B), comparison of the entire ensemble reveals significant structural differences in this area albeit with a more compact character distance-wise than would be expected in the absence of the disulfide (Fig. 3.1C).

Table 3.1 Statistics for SPIN-CYS NMR solution structure^a.

PDB Accession Code	9BD0
NOE-based distance restraints	
Intra-residue ($i = j$)	134
Sequential ($ i-j = 1$)	321
medium range ($1 < i-j < 5$)	281
Long Range ($ i-j \geq 5$)	56
Total	792
Hydrogen bond distance restraints	10
Dihedral angle restraints (Φ and Ψ)	100
Restraint Violations	
NOE $>0.2 \text{ \AA}$	11.85
NOE $>0.5 \text{ \AA}$	0.25
Dihedral angle $>5^\circ$	0

PDB Accession Code	9BD0
RMSD from the mean structure (Å) ^a	
Main chain	0.88 ± 0.29
Heavy atoms	1.52 ± 0.32
RMSD from experimental restraints	
NOE-based distance restraints (Å)	0.032 ± 0.001
Dihedral angle restraints (°)	0.683 ± 0.114
Deviations from ideal geometry	
RMS deviation for bond angles (°)	0.2
RMS deviation for bond lengths (Å)	0.001
Ramachandran analysis (PROCHECK-NMR)	
Most favored regions (%)	88.6
Additionally allowed regions (%)	10.2
Generously allowed regions (%)	1.2
Residues in disallowed regions	0
Protein Structure Validation Suite Analyses ^c	
PROCHECK G-factor (all dihedral)	−0.90 N/A (−5.32)
PROCHECK G-factor (phi/psi)	−0.35 N/A (−1.06)
Verify3D	0.17 ± 0.03 (−4.65)
ProsaII (-ve)	0.31 ± 0.11 (−1.41)
MolProbity clashscore ^d	14.21 ± 5.84 (−0.91)
MolProbity clashcore Analysis ^e	26.60

a- Statistics presented for the 20 lowest energy structures of the ensemble.

b- Calculated using Cyana 2.1.

c- Values in parentheses are Z-scores as calculated by version 1.5.

d- Clashscore obtained from the PSVS server.

e- Clashscore obtained from Molprobity server version 4.5.1.

To further explore the structural differences between SPIN-CYS and wild-type *S. aureus* SPIN (hereafter SPIN-WT) in this N-terminal region, we compared the HetNOE values and relaxation rates (i.e. R2/R1) across the polypeptide backbone of the two proteins (10). Whereas the average HetNOE value within the 13-residue N-terminal region of SPIN-CYS is 0.482 (Fig.

3.2A), the average value within the same residues of SPIN-WT is considerably lower at 0.169 (Fig. 3.2B). By contrast, the average HetNOE values across the ~60-residue helical bundle domain of SPIN-CYS and SPIN-WT are in good agreement with one another at 0.788 and 0.786, respectively. Similarly, the average R2/R1 ratio within the N-terminal region of SPIN-CYS is 3.057 (Fig. 3.2C), but is 2.512 for the corresponding region of SPIN-WT (Fig. 3.2D). The average R2/R1 values across the helical bundle domains of SPIN-CYS and SPIN-WT are 3.923 and 3.614, respectively. Considering these data in total, the observed increase in HetNOE and enhanced relaxation ratio (R2/R1) in the N-terminus of SPIN-CYS relative to SPIN-WT strongly suggests that the introduction of the disulfide greatly restricted the overall conformational flexibility of the N-terminus even though the structure solution itself does not reveal the presence of a β -hairpin as initially anticipated.

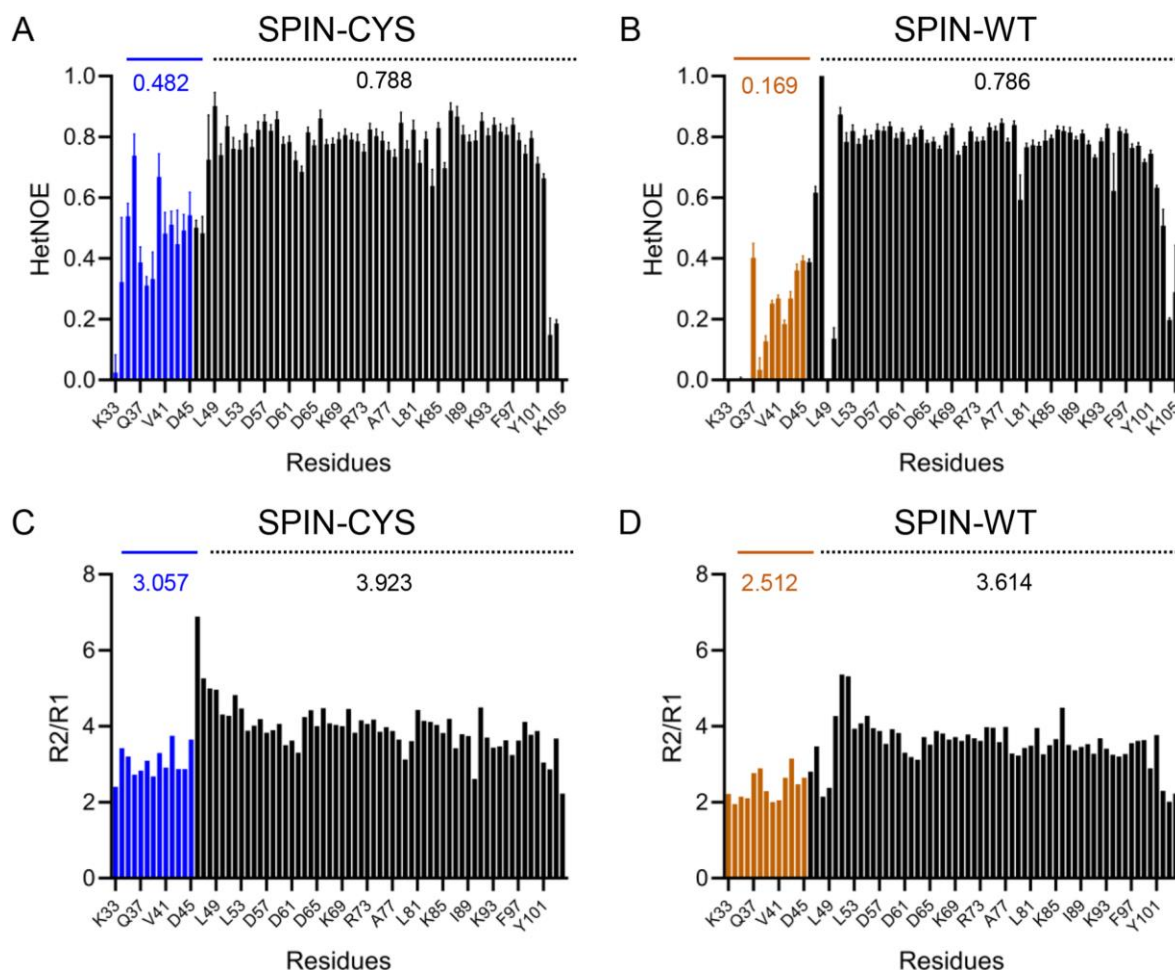


Figure 3.2 Solution Dynamic Properties of SPIN Proteins as Assessed by NMR. (A) Residue-level hetNOE values for SPIN-CYS.

Values for residues comprising the N-terminal region are shown in blue, while those within the C-terminal α -helical bundle domain are shown in black. Average values within each region appear at the top of the panel. (B) Identical information to Panel A, except for the SPIN-WT protein. Here, the values for residues comprising the N-terminal region are shown in orange. (C) Residue-level values for the ratio of observed ^{15}N -R2 (transverse relaxation rate) and ^{15}N -R1 (longitudinal relaxation rate) for SPIN-CYS. Values for residues comprising the N-terminal region are shown in blue, while those within the C-terminal α -helical bundle domain are shown in black. Average values within each region appear at the top of the panel. (D) Identical information to Panel C, except for the SPIN-WT protein. Here, the values for residues comprising the N-terminal region are shown in orange.

3.2. Disulfide Insertion in the N-terminus of *S. aureus* SPIN Increases its Inhibitory Potency Against MPO

We continued our characterization of SPIN-CYS to examine whether the constrained structural properties of its N-terminus might influence function of this protein as an MPO inhibitor.

As an initial assessment, we compared the potency of SPIN-CYS versus that of SPIN-WT in a chromogenic assay that measures the peroxidase cycle of human MPO (13, 20, 21). We performed assays in triplicate across a two-fold dilution series of SPIN proteins ranging from ~32 μ M to 1 nM and fit the observed MPO activity to a four-parameter dose-response curve to determine apparent IC₅₀ values and the associated confidence intervals (Fig. 3.3A & Table 3.2). Interestingly, we observed an enhancement of MPO inhibition for SPIN-CYS (IC₅₀ 3.6 nM) when compared to SPIN-WT (IC₅₀ 6.6 nM). Although this enhancement is only roughly ~1.8-fold, the associated confidence intervals derived from curve fitting do not overlap with one another, suggesting that the observed change potency is significant (Table 3.2). To further validate the accuracy of these findings, we compared the potencies of three SPIN orthologs from *S. delphini*, *S. schleiferi*, and *S. intermedius* in the same assay since these proteins had been reported to exhibit weaker dose-dependent inhibition of human MPO in a previously used method for assessing the MPO peroxidase cycle (7) (Fig. 3.3A). Consistent with our previously published observations, we found that SPIN-delphini (IC₅₀ 44 nM) is ~6.5-fold weaker than SPIN-WT, while both SPIN-schleiferi (IC₅₀ 102 nM) and SPIN-intermedius (IC₅₀ 125 nM) are weaker still than SPIN-delphini but comparable to one another (7). Thus, we concluded that the difference observed between SPIN-CYS and SPIN-WT represents a *bona fide* change in potency.

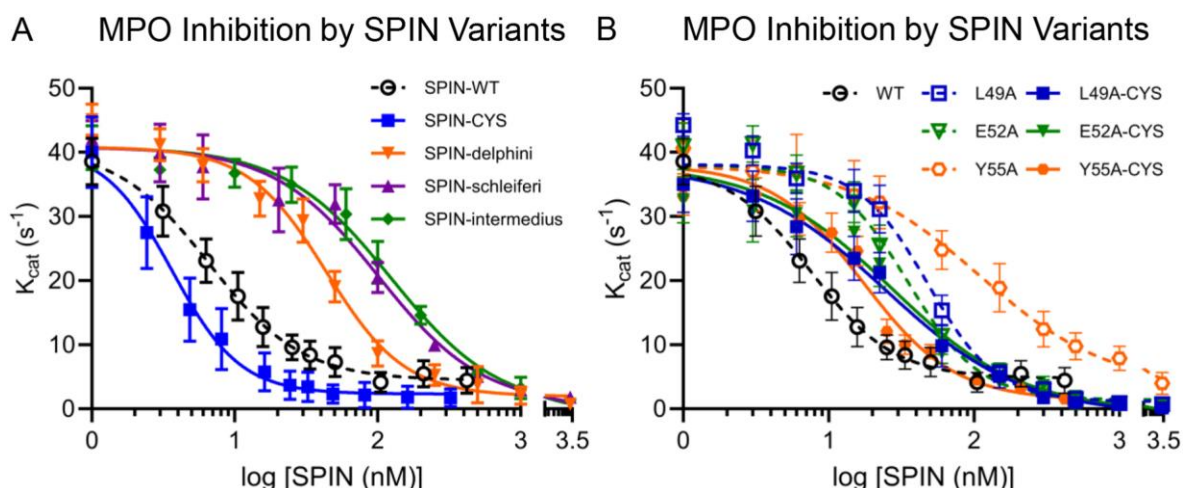


Figure 3.3 MPO Inhibitory Properties of Various *S. aureus* SPIN Mutants.

(A) The inhibitory properties of SPIN-CYS were compared to SPIN-WT, SPIN-delphini, SPIN-schleiferi, and SPIN-intermedius in an enzyme assay designed to interrogate the peroxidase cycle of MPO. A minimum of nine independent replicates were conducted on each inhibitor prior to fitting the respective data to a four-parameter dose-response inhibition curve. The outcome of curve fitting is shown in Table 3.2. Data points are shown as the mean plus or minus the standard deviation. A legend is inset. (B) Identical experiment to that shown in Panel A, except that SPIN proteins with deleterious mutations in the α -helical bundle domain were investigated within either the SPIN-CYS (solid lines) or the SPIN-WT (dashed lines) framework. A legend is inset.

Table 3.2 Functional properties of SPIN variants as assessed by enzyme assay and surface plasmon resonance^{a,b}.

SPIN Variant	IC ₅₀ (nM) ^a	K _D (nM)	k _{at} (M ⁻¹ s ⁻¹) × 10 ⁴	k _{dt} (s ⁻¹) × 10 ⁻³	k _{a2} (s ⁻¹) × 10 ⁻³	k _{d2} (s ⁻¹) × 10 ⁻³
SPIN-WT ^c	6.6 (6.2-7.1)	44.8 ± 9.5	22.6 ± 3.0	22.2 ± 11.7	5.2 ± 3.7	4.6 ± 1.6
SPIN-dN ^{c,d}	n.d.	48.9 ± 4.2	20.8 ± 1.6	10.2 ± 0.8	-	-
SPIN-CYS ^c	3.6 (3.4-3.9)	25.2 ± 2.8	40.2 ± 6.1	24.7 ± 7.4	9.5 ± 4.8	6.4 ± 1.2
L49A ^e	37.8 (34.3-41.8)	287.0 ± 31.7	2.5 ± 0.4	20.2 ± 2.9	3.8 ± 0.3	2.1 ± 0.3
L49A-CYS	29.1 (25.3-33.9)	211.8 ± 58.5	3.8 ± 1.3	25.0 ± 5.7	9.0 ± 1.4	3.3 ± 0.2
E52A ^e	64.4 (58.9-70.2)	492.0 ± 56.2	1.7 ± 0.4	18.6 ± 5.5	4.1 ± 1.3	3.2 ± 1.0
E52A-CYS	33.5 (30.0-37.7)	345.2 ± 34.9	1.5 ± 0.5	15.0 ± 6.8	4.7 ± 1.7	2.1 ± 1.0
Y55A ^e	181.1 (126.5-321.5)	n.d.	n.d.	n.d.	n.d.	n.d.
Y55A-CYS	83.3 (71.1-100.9)	11590 ± 1200	0.05 ± 0.01	19.6 ± 5.7	2.6 ± 1.1	1.2 ± 0.3

a- Values in parentheses represent the lower and upper limits for the 95 % confidence interval following non-linear curve fitting.

b- Aside from IC₅₀, all other values are reported as the mean ± standard deviation across replicate measurements (n > 4).

c- SPR parameters determined from experiments using MPO obtained from Elastin Products Corp.

d- Due to the lack of the N-terminal region, these data were analyzed with a single site kinetic model.

e- Values taken from a recent study by Fatehi et al. for the sake of comparison (13).

Because the absolute change in potency resulting from the disulfide insertion is modest, we sought to further substantiate its influence on SPIN function. In that regard, we recently completed a residue-level analysis of the α -helical bundle domain of SPIN-WT that identified several residues, including L49, E52, and Y55, required for maximal MPO binding and inhibition (13). We therefore prepared a series of site-directed mutants wherein the L49A, E52A, and Y55A mutations were incorporated into the SPIN-CYS framework. We then compared the properties of these new proteins with the L49A, E52A, and Y55A mutants that lacked the disulfide insertion (Fig. 3.3B & Table 3.2). Consistent with our data on SPIN-CYS versus SPIN-WT, we found that the N-terminal disulfide results in a ~ 1.3 -fold gain for L49A-CYS (IC_{50} 29.1 nM), a ~ 1.9 -fold gain for E52A-CYS (IC_{50} 33.5 nM), and ~ 2.2 -fold gain (IC_{50} 83.3 nM) in inhibitory potency for Y55A-CYS. Thus, the disulfide insertion also provides a gain of function in these SPIN variants despite the presence of the otherwise deleterious mutations.

3.3. Disulfide Insertion Increases Potency by Increasing the Apparent Affinity of the Inhibitor with MPO.

In our earlier study of the SPIN α -helical bundle domain, we found that the L49A, E52A, and Y55A mutants are diminished in MPO inhibition due to a reduction in their apparent affinities for MPO (13). Therefore, we decided to investigate whether the increased potency of SPIN-CYS against human MPO is due to an increased apparent affinity when compared to SPIN-WT. We used SPR to compare binding of SPIN-CYS, SPIN-WT, and the N-terminal deletion mutant (i.e. SPIN-dN) to immobilized human MPO (Fig. 3.4A-C & Table 3.2). Interestingly, we found that SPIN-CYS (K_D 25.2 nM) displays an ~ 1.8 -fold increase in apparent affinity when compared to SPIN-WT (K_D 44.8 nM). Similarly, we found that the N-terminal disulfide results in an overall increase in apparent affinity for the mutants utilized above. Both the L49A-CYS (K_D 211.8 nM) and E52A-CYS (K_D 345.2 nM) mutants display an ~ 1.4 -fold increase in apparent affinity for

human MPO over the L49A and E52A mutants (Fig. 3.4D-E & Table 3.2). Since Y55A within the SPIN-WT framework fails to bind human MPO in SPR experiments (13), a quantitative comparison of apparent affinities could not be made between the Y55A mutants; however, Y55A within the SPIN-CYS framework does show evidence of binding MPO on its own (Fig. 4.4F & Table 3.2). Considered as a whole, these observations show that the N-terminal disulfide results in an increase in apparent affinity for MPO when compared to wild-type SPIN.

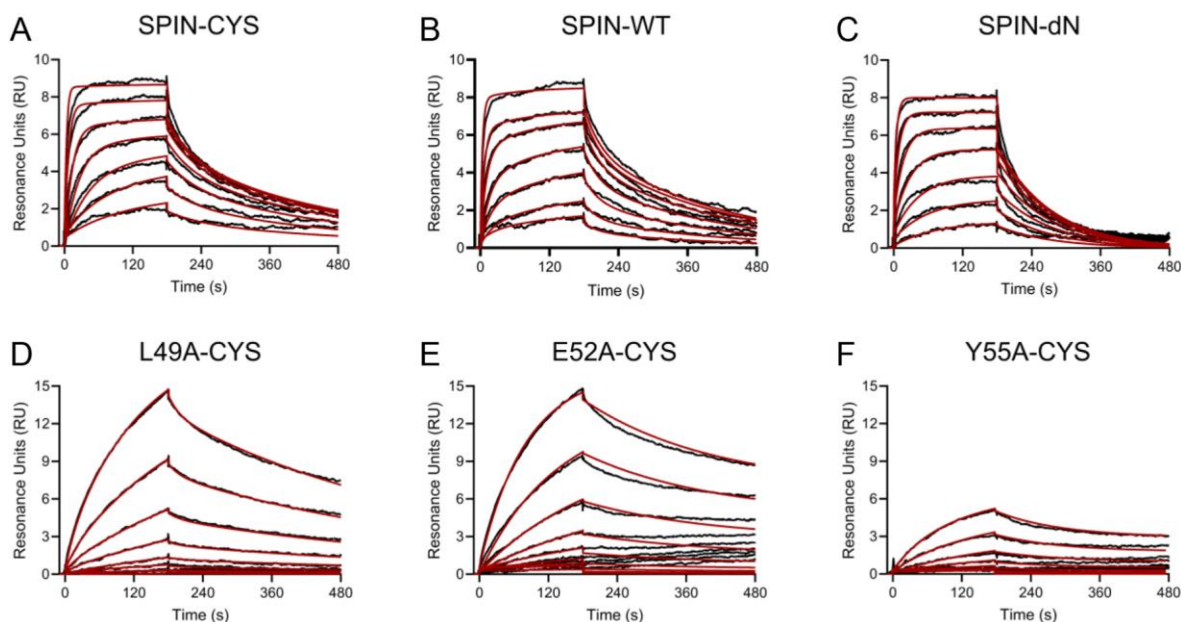


Figure 3.4 MPO Binding Properties of *S. aureus* SPIN Mutants as Determined by SPR.

The binding properties of SPIN-CYS to immobilized human MPO were compared to SPIN-WT and various mutants. Reference-subtracted sensorgrams (black traces) were obtained for each sample following injection of a two-fold dilution series, where 1 μ M was used as the highest concentration for each analyte. Sensorgram series were fitted globally to a two-state binding model (red traces). The outcome of fitting is shown in Table 3.2. (A) SPIN-CYS (B) SPIN-WT (C) SPIN-dN (D) L49A-CYS (E) E52A-CYS (F) Y55A-CYS.

Earlier characterization of certain SPIN mutants revealed that their observed loss of inhibitory potency correlates directly with loss of apparent affinity for MPO (13). Further examination of the rate constants governing their interaction with MPO revealed that this loss of apparent affinity is due primarily to slowing the association rate of these SPIN variants with MPO.

Therefore, we wondered whether the increases in apparent affinity observed for the SPIN-CYS proteins are due to an increase in the association rate with MPO relative to their SPIN-WT counterparts. To address this question, we compared the apparent affinities and various rate constants derived from fitting experimental sensorgram series to identify potential trends in the data (Fig. 3.5 & Table 3.2). It should be noted that SPIN-CYS and SPIN-WT were fit to a two-state binding model owing to the conformational change that occurs in the SPIN N-terminal region following initial binding (11, 13) (Fig. 3.5A), while the sensorgram series from the SPIN-dN mutant was fit to a single-state binding model as this protein lacks the SPIN N-terminal region(8). Examination of the apparent affinities (Fig. 3.5B) and individual rate constants (Fig. 3.5C-F) revealed that the enhanced affinity of SPIN-CYS seems to arise from an increase in the association rates attributable to both the initial binding of the helical bundle domain (i.e. the second order rate constant, k_{a1} ; ~2.4-fold increase) as well as the conformational change of the N-terminus (i.e. the first order rate constant, k_{a2} ; ~1.8-fold increase). This is partly offset by an increase in the dissociation rate of the conformational change (i.e. the first order rate constant, k_{d2} ; ~1.4-fold increase). Similarly, the enhanced affinity of L49A-CYS appears due to an increase in both association rate constants (k_{a1} ~1.5-fold increase; k_{a2} ~2.4-fold increase) that is partly offset by an increase in the second dissociation rate constant (k_{d2} ~1.6-fold increase). Although E52A-CYS displayed enhanced affinity when compared to E52A alone (Table 3.2), any changes to rate constants could not be discerned due to too much variability in the data. Despite this limitation, it seems that incorporation of a disulfide bond in the N-terminal region of SPIN provides an enhancement in potency and affinity that arises from an increased association rate of the SPIN protein with MPO.

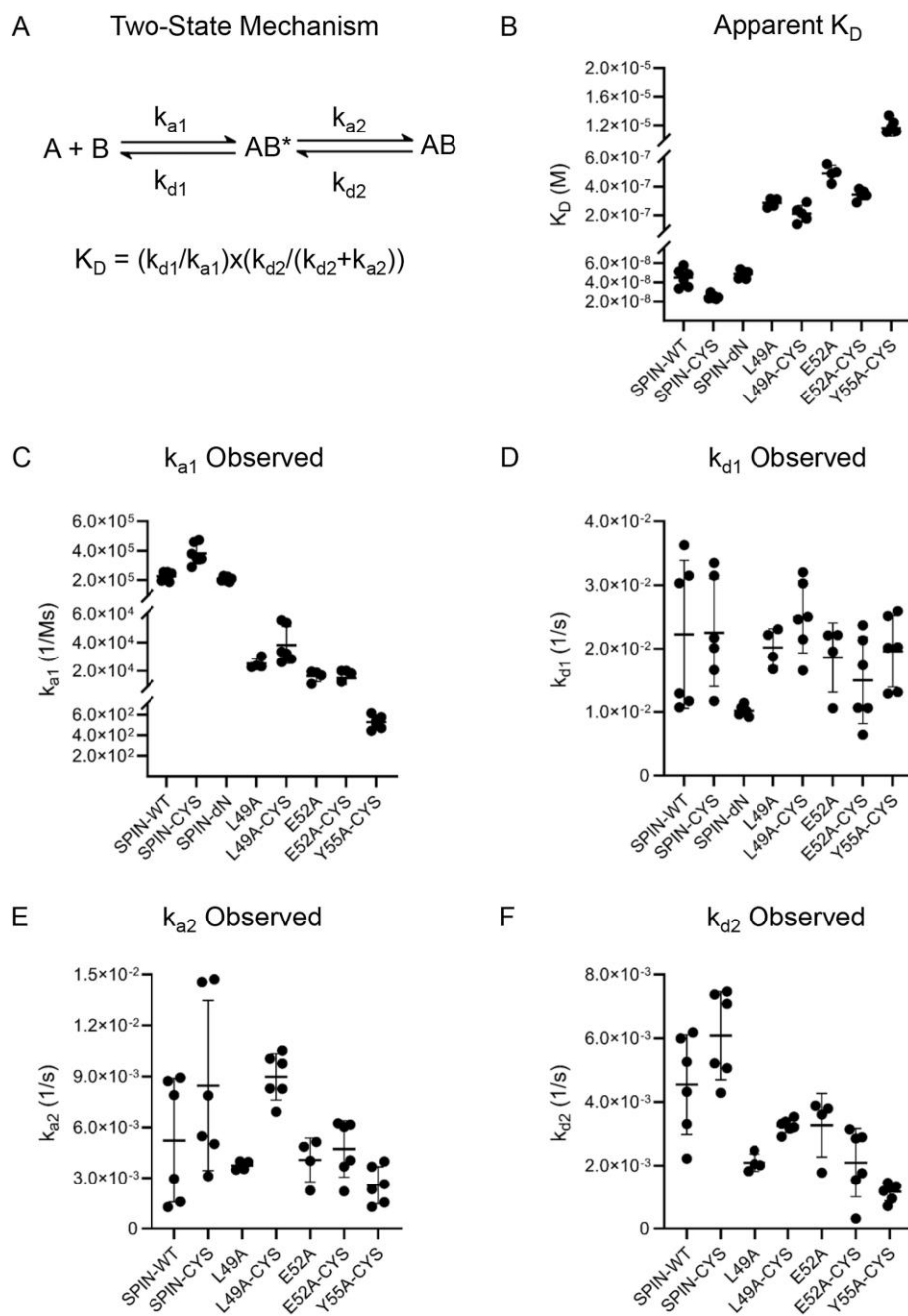


Figure 3.5 Analysis of the MPO-Binding Parameters for Various *S. aureus* SPIN Mutants.

(A) Description of the two-state binding mechanism used to analyze interaction of SPIN with immobilized human MPO. Value k_{a1} is a second order rate constant, while values k_{d1} , k_{a2} , and k_{d2} are first order rate constants. The equation for the apparent K_D appears below the mechanism. (B) Comparison of apparent K_D values for SPIN-WT and various SPIN mutants. Error bars show the mean, plus or minus the standard deviation for a minimum of four replicates. (C) Observed k_{a1} for SPIN-WT and various mutants. (D) Observed k_{d1} for SPIN-WT and various mutants. (E) Observed k_{a2} for SPIN-WT and various mutants. (F) Observed k_{d2} for SPIN-WT and various mutants.

Discussion

Although the molecular plug model has been a facile way of explaining MPO inhibition by SPIN, emerging evidence has challenged that notion by showing that SPIN only partly blocks halide oxidation (11). This has raised questions about the SPIN N-terminus and the events that lead to its change from a disordered structure to a folded β -hairpin that inserts into the MPO active site channel. Because separate computational studies have proposed that this binding and folding event is highly cooperative (12), we investigated whether changing the structural properties of this otherwise disordered SPIN N-terminus would impact its ability to inhibit MPO. Our strategy was to insert a stabilizing disulfide bond into the SPIN N-terminus by targeted mutations in two residues, V34 and D44, whose sidechains lie very near one another in the structure of MPO-bound *S. aureus* SPIN (6). We succeeded in forming a disulfide bond within the SPIN-CYS N-terminus (Fig. 3S1); however, solution structure calculation on this mutant revealed that the N-terminus did not adopt a defined β -hairpin, but rather an ensemble of lariat-like conformations whose overall dimensions and dynamics were constrained by the presence of the disulfide (Figs. 3.1-3.2, Fig. 3S1 & Table 3.1). We found that this disulfide caused a modest yet statistically meaningful gain in potency of SPIN-CYS against human MPO when compared to SPIN-WT (Fig. 3.3 & Table 3.2). We also showed that this increase was retained in the presence of otherwise deleterious mutations (Fig. 3.3 & Table 3.2). Using SPR, we determined that the increase in potency of each disulfide-containing mutant arises from increasing its apparent affinity for MPO, apparently through enhancement of its association rate with MPO (Figs. 3.4-3.5 & Table 3.2). Therefore, we conclude that restricting the conformational space accessible to the SPIN N-terminus promotes binding to MPO, leading to the gain of function we observed in these experiments. In the future, it may prove

interesting to test if the increased potency of these disulfide-containing mutants is also retained in assays that interrogate the halogenation cycle of MPO.

Inserting a disulfide had the intended effect of restricting the conformational space accessible to the SPIN N-terminus, however, it failed to trap this region in the β -hairpin structure seen in the MPO-bound inhibitor. There may be at least two plausible explanations for this observation. On one hand, the β -hairpin may result from simple spatial restrictions imposed on SPIN by the MPO active site channel when the two proteins interact. On the other hand, the β -hairpin could also arise through formation of key hydrogen bonds between groups from the SPIN N-terminus and others from the MPO active site channel. In either scenario, the presence of the binding partner (in this case, MPO) would necessarily play such a fundamental role in folding the inhibitory N-terminus that it cannot be replicated by trivial modifications of the SPIN protein alone. It might be informative, however, to investigate alternative locations for disulfide insertions and assess their consequences. One such option is S36 and V41, whose sidechains are similarly distant to those of V34 and D44. Interestingly, preliminary characterization of an S36C/V41C disulfide insertion revealed that this mutant was ~ 1.5 -fold diminished in potency compared to SPIN-WT (Fig. 3S2). Another possibility would be a combinatoric mutant V34C/S36C/V41C/D44, which would result in two pairs of disulfide bonds within the N-terminus. Presumably, the second disulfide bond would further constrain the SPIN N-terminus within conformational space and perhaps lead to additional gain of function. Unfortunately, incorporating four reactive cysteine residues within such a small region of polypeptide could potentially cause problems like incorrect and/or partial disulfide bonding. While these concerns highlight the limitations of the approach we used in this study, it still provided useful insights into how the structure of the SPIN N-terminus influences its function.

There are several orthologs of *S. aureus* SPIN that also bind and inhibit human MPO (7, 9). The best characterized of these is SPIN from *S. delphini* (7), which inhibits human MPO ~6.5-fold less effectively than *S. aureus* SPIN and which we utilized here as a control for reproducibility of our assay (Fig. 3.3). SPIN-delphini is 53% identical to SPIN-aureus, and their overall structures when bound to recombinant human MPO are essentially identical (7). Yet, recent computational studies by Zhang et al. suggest that the mechanisms underlying the coupled binding and folding of their respective N-termini are fundamentally different (12). Whereas the N-terminus of SPIN-aureus obeys a highly cooperative binding and folding process, the same region of SPIN-delphini seems to follow instead a conformational selection mechanism (12). Zhang et al. further observed that the SPIN-delphini N-terminus has a much greater tendency to adopt β -hairpin-type structures in the absence of MPO (12). During our initial characterization of SPIN-delphini we found that the weaker affinity of this ortholog for human MPO is largely due to a slower association rate with MPO when compared to SPIN-aureus (7). Intriguingly, these data seem to argue that preforming β -hairpin structures like those seen in the MPO-bound form of SPINs may be counterproductive to inhibitor binding. In the early stages of this work, we attempted to create a disulfide-insertion variant of SPIN-delphini to serve as a comparator for SPIN-CYS; unfortunately, that protein was prone to incomplete intramolecular disulfide bond formation, which precluded further study. An alternative approach for investigating this issue was to create a chimeric protein wherein the SPIN-delphini N-terminus was appended to the SPIN-aureus α -helical bundle domain. We hypothesized that this chimera ought to have a reduced potency for human MPO due to a slower association rate, presumably resulting from a preformed β -hairpin character in its N-terminal region. Indeed, when we prepared and preliminarily characterized a SPIN-delphini-aureus chimera, we found that its potency was ~5-fold diminished when compared to SPIN-WT (Fig. 3S2). Although we did not

perform a detailed structural analysis on this chimera, our observations support the idea that the reduced potency of SPIN-delphini may be partly due to the preformed β -hairpin character of its N-terminus.

Considering the information in the previous paragraph, we may have been fortunate that our disulfide insertion in SPIN-aureus resulted in a more physically constrained ensemble rather than a fixed β -hairpin *per se*. Although the gain in function of SPIN-CYS was modest, we found that it was reproducible not only within individual experiments, but across various assays for SPIN activity (e.g. MPO inhibition vs MPO binding). The gain of function we observed for SPIN-CYS could be attributed to an increase in its affinity for MPO that appeared commensurate with the increase in its association rate (Table 3.2). We suspect this occurs because the SPIN-CYS protein is more physically compact than SPIN-WT when it first encounters MPO, leading to an increased rate of productive binding events. Although that is largely conjecture, the gain of function in terms of potency, affinity, and association rate was comparable across a panel of SPIN-CYS mutants that had distinct deleterious mutations within their α -helical bundle domains. How these experimental observations correlate with computational studies suggesting a highly cooperative binding and folding mechanism of SPIN-aureus is not clear at this time (12). Nevertheless, our previous observations on the H43A/D44A/D45A mutant of SPIN-aureus that binds MPO with wild-type affinity but fails to inhibit its activity strongly suggest that the N-terminal region requires a guide to fold into the MPO active channel (8). In the future, it might prove informative to examine mutations in H43 and D55 within the SPIN-CYS framework to further investigate this issue and how it is influenced by the conformational properties of the SPIN N-terminus.

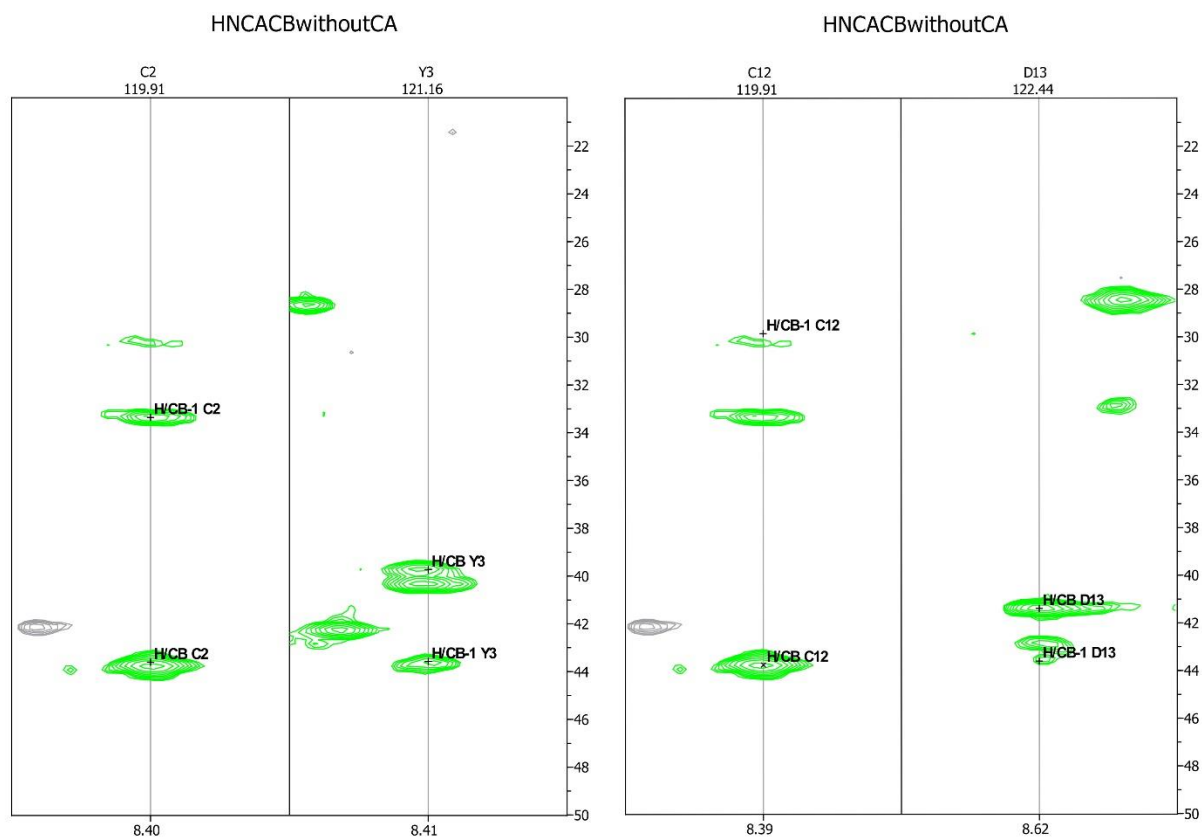


Figure 3S1 Confirmation of a Disulfide Bond between C34 and C44 in the SPIN-CYS Protein.

Visualization of segments within the HNCACB spectrum collected on a sample of $^{15}\text{N}/^{13}\text{C}$ -labeled SPIN-CYS protein. The chemical shift values of the $\text{C}\beta$ resonances for C34 (left panel, referred to therein as C2; 43.6 ppm) and C44 (right panel, referred to therein as C12; 43.5 ppm) correspond well to the expected value of about 43 ppm (^{13}C) if the residue participates in a disulfide bond. For a reduced cysteine, the expected value for the $\text{C}\beta$ resonance is about 29 ppm. The peak annotated as H/CB-1 represents the $\text{C}\beta$ resonance for the previous residue in both panels.

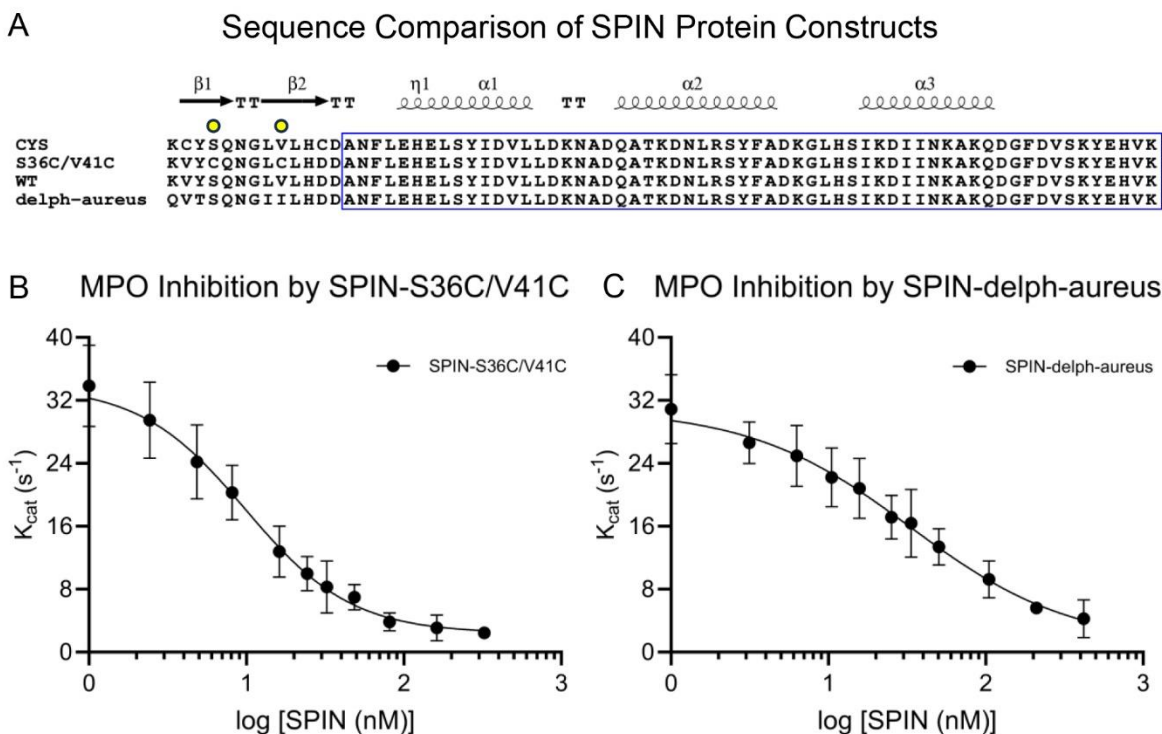


Figure 3S2 Sequence Information and Partial Characterization of Two Additional SPIN Mutants.

(A) Sequence alignment of wild-type SPIN-aureus, SPIN-CYS, an S36C/V41C disulfide-insertion mutant and a chimeric SPIN consisting of the N-terminal region from SPIN-delphini and the C-terminal region of SPIN-aureus. Secondary structure elements of the MPO-bound form of *S. aureus* SPIN are shown above the sequence alignment, where the residues corresponding to the α -helical bundle domain are enclosed in a blue rectangle. The locations of positions 36 and 41 are highlighted with yellow circles. (B) The inhibitory properties of SPIN-S36C/V41C were investigated using an MPO activity assay. 15 independent replicates were conducted prior to fitting the respective data to a four-parameter dose-response inhibition curve. The outcome of curve fitting is shown as a solid line, while the data points are shown as the mean plus or minus the standard deviation. The apparent IC_{50} value is 9.9 nM (8.8 - 11.2 nM). (C) The inhibitory properties of SPIN-delph-aureus were investigated using an MPO activity assay. 15 independent replicates were performed prior to curve fitting. The apparent IC_{50} value is 33.1 nM (25.1 – 48.9 nM).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

ACKNOWLEDGEMENTS

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Chapter 4 - ^1H , ^{15}N , and ^{13}C Backbone Resonance Assignments of the C4b-Binding Region from the *S. aureus* Extracellular Adherence Protein

This chapter has been published as a journal article in the journal of Biomolecular NMR assignments

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Abstract (201 words)

The Extracellular Adherence Protein (Eap) from *Staphylococcus aureus* is a potent inhibitor of the classical and lectin pathways of the complement system. Previous studies have shown that Eap binds with nanomolar affinity to complement component C4b and prevents C4b binding the pro-protease, C2, thereby inhibiting formation of the pro-C3 convertase shared by the classical and lectin pathways (1). The C4b-binding and complement-inhibitory properties of Eap from *S. aureus* strain Mu50 lie within the two C terminal-most Eap domains (i.e. Eap34) (1). Interestingly, Eap34 binds C4b with an apparent K_D that is nearly 100-fold tighter than that of either Eap3 or Eap4 alone (2). This suggests that linking these two domains into a single molecule is a significant determinant of Eap function. To better understand this property at the structural level, we undertook a solution NMR study of the ~23 kDa Eap34 protein. In this communication, we report that greater than 98% of the total non-proline backbone residues have been assigned. These data have been deposited in the BMRB database under the accession number 50210.

Keywords

Staphylococcus aureus, Extracellular adherence protein, NMR Resonance Assignment, virulence protein.

Biological context

The pathogenic Gram-positive bacterium *Staphylococcus aureus* secretes an array of proteins that disrupt function of the human innate immune system (3) by inhibiting the central processes of opsonization, recruitment of neutrophils, and subsequent phagocytosis (3–7). The complement system plays a key role in each of these events, and well over a dozen different secreted proteins from *Staphylococcus aureus* have been shown to interfere with complement activity. Woehl et al. recently characterized the *Staphylococcus aureus* Extracellular Adherence Protein (Eap) as a novel inhibitor of both the classical (CP) and lectin (LP) complement pathways (1). Eap binds with nanomolar affinity to complement component C4b, inhibits formation of the CP and LP pathway pro-C3 convertase that assembles on C4b, and thereby blocks generation of the fully-active C3 convertase that is shared by these two most potent routes for initiating complement activity. Consequently, Eap plays an important role in inhibiting complement-driven opsonization of *Staphylococcus aureus* cells by the activated fragment of C3, known as C3b.

Eap is a ~50-70 kDa protein comprised of multiple repeating domains of ~100 residues depending upon the specific isoform examined (8). Although each domain is connected to the adjacent domain with a short (~10 residues) linker region, previous studies have suggested that adjacent domains may interact with each other specifically (9). Domain deletion studies on the four-domain isoform of Eap expressed by *Staphylococcus aureus* strain Mu50 demonstrated that the CP/LP inhibitory activity of Eap lies within its two C terminal-most repeats (1). Indeed, the truncated form of the protein consisting of domains 3 and 4 alone (i.e. Eap34) binds with nanomolar affinity to complement component C4b (1) ($K_D=525$ nM) and retains CP/LP inhibitory activity comparable to the full length Eap protein. By contrast, Eap domains 1 and 2 (i.e. Eap12) bind only weakly to C4b and inhibit the CP/LP to only a minor extent. Interestingly, while the

individual domains represented by Eap3 and Eap4 bind C4b with K_D values $\sim 40 \mu\text{M}$ (2) their affinity is almost 100-fold weaker than that of Eap34. This suggests that the tethering of these domains to one another via a linker region is an important determinant for function of this protein. Structural studies of Eap34 should therefore be useful in understanding how this occurs.

Here, we report backbone ^1H , ^{15}N , and ^{13}C resonance assignments for Eap34 from *S. aureus* strain Mu50. These resonance assignments will help us toward determination of residues in Eap34 protein involved in binding with ligand and better understand the interaction of Eap34 with C4b.

Methods and experiments:

Protein expression and purification:

Recombinant Eap34 from *S. aureus* strain Mu50 was overexpressed in minimal medium (M9) enriched with $^{15}\text{NH}_4\text{Cl}$ (1gram/litre) and ^{13}C -glucose (1g/litre) and purified from *E. coli* strain BL21(DE3) according to the general methods described previously (10) , with the exception that protein expression was carried out at 18 °C. The targeted protein was isolated from the soluble fraction of the bacterial cell homogenate by NiNTA-affinity chromatography carried out under native conditions. Following initial purification, the fusion tag was removed by site-specific cleavage using Tobacco Etch Virus protease as reported earlier (10). Final purification was achieved by gel-filtration chromatography using a Superdex 75 26/60 column (GE Healthcare). The purified protein was buffer exchanged into doubly distilled H_2O prior to lyophilization. Purification of ^{15}N - and $^{15}\text{N}/^{13}\text{C}$ -enriched protein samples of Eap34 were carried out as described above using the methods for expression and purification developed in our laboratory and published earlier (11).

NMR spectroscopy

The samples for NMR experiments were prepared using 0.5 – 0.75 mM uniformly ^{15}N or $^{13}\text{C}/^{15}\text{N}$ double-labeled Eap34 protein in 50 mM sodium phosphate buffer (pH 6.5) containing 50 mM NaCl, and 5 % (v/v) D_2O (used as a lock solvent). The purity and mass of the uniformly ^{15}N or $^{13}\text{C}/^{15}\text{N}$ double-labeled protein were verified using mass spectrometry (Ultra Flex III TOF, Bruker Daltonics) prior to NMR data acquisition. All NMR spectra were acquired at 25°C on Agilent 700 MHz NMR Spectrometer equipped with a 5 mm inverse detection, cryogenically cooled, HCN triple resonance probe with Z-axis pulse field gradient operating at 700 MHz for ^1H frequency. Backbone assignments were achieved using 2D ^1H - ^{15}N HSQC and 3D HNCO, HN(CA)CO, HN(CO)CA, HNCA and HNCACB NMR experiments. The three-dimensional NMR experiments were acquired using non-uniform sampling. For 3D NMR data acquisition, 12% of the total points in the Nyquist grid indirect dimension were sampled non-uniformly using a Poisson-Gap sampling scheme and the spectra were reconstructed using hmsIST (12). The acquisition parameters for all the NMR experiments are mentioned in Table 4.1. All spectra were processed using NMRpipe (13). Eap34 backbone resonances were assigned following standard procedures. The spectral analyses were done using CARA (14) (<http://cara.nmr-software.org/portal/>). The reference for the ^1H chemical shift assignments was relative to the ^1H chemical shift of 2,2-dimethyl-2-silapentane-5-sulphonic acid (DSS) at 25°C. The ^{13}C and ^{15}N chemical shift references were indirectly calculated to DSS, using the absolute frequency ratios.

Sequence specific resonance assignments are indicated by single letter amino acid residue name and sequence number of the Eap34 construct.

Extent of assignments and data deposition

A 2D ^1H - ^{15}N HSQC NMR spectrum of Eap34 with resonance assignments is presented in Figure 4.1. In this spectrum the chemical shifts in the ^1H dimension is well dispersed suggesting that Eap34 is a highly structured protein. The amino acid numbering was based upon the Eap34 sequence which is directly given from the Eap34 expression construct followed by TEV cleavage. The unassigned/unpicked resonances in Figure 4.1 were from the side chain of amino acids. There were 5 proline residues which could not be assigned by triple resonance backbone experiments. A standard sequential assignment procedure based on pairs of triple resonance experiments using HNCA, HNCO, HNCACB, HNCOCA and HNCACO spectra (15, 16) was followed to complete 98.0% of total non-proline residues backbone assignments. The percentage of NH, C- α , C- β and CO chemical shift assignments were 98.0%, 97.0%, 93.1% and 96.5% respectively. Most of the unassigned residues were located in the linker and the N-terminal region of Eap34 and showed severe overlapping, broadening or were otherwise difficult to distinguish from noise signals in the spectra. The ^1H , ^{15}N , and ^{13}C chemical shift values for Eap34 have been deposited at the BioMagResBank (<http://www.bmrb.wisc.edu/>) under BMRB accession number 50210.

The secondary structure elements were predicted by the TALOS-N webserver (17) using C- α , C- β , CO, N and HN chemical shift values of Eap34. Figure 4.2A represents dihedral angles of Eap34 and figure 4.2B depicts the α -helices and β -strands probability predicted by TALOS-N. We have also compared the chemical shift values for domain 3 and 4 residues in Eap34 with previously assigned chemical shift values of corresponding residues in Eap3 and Eap4 (11, 18). Eap3 and Eap 4 sequences start with extra three residues (GST) compared to Eap34 sequence. We

have improvised this 3 residue offset correction in deriving the chemical shift differences presented in Figure 4.3. A bar plot of the chemical shift difference is shown in Figure 4.3. We calculated the chemical shift difference for corresponding residues among Eap34, Eap3 and Eap4 as described earlier (19) where the total change in chemical shift (ppm) for residues was carried out using the equation $\sqrt{(25(\Delta^1\text{Hppm})^2 + (\Delta^{15}\text{Nppm})^2)}$. We observed some regions of Eap34 having minor chemical shift differences. Interestingly our secondary structure prediction is well matched with Eap domains.

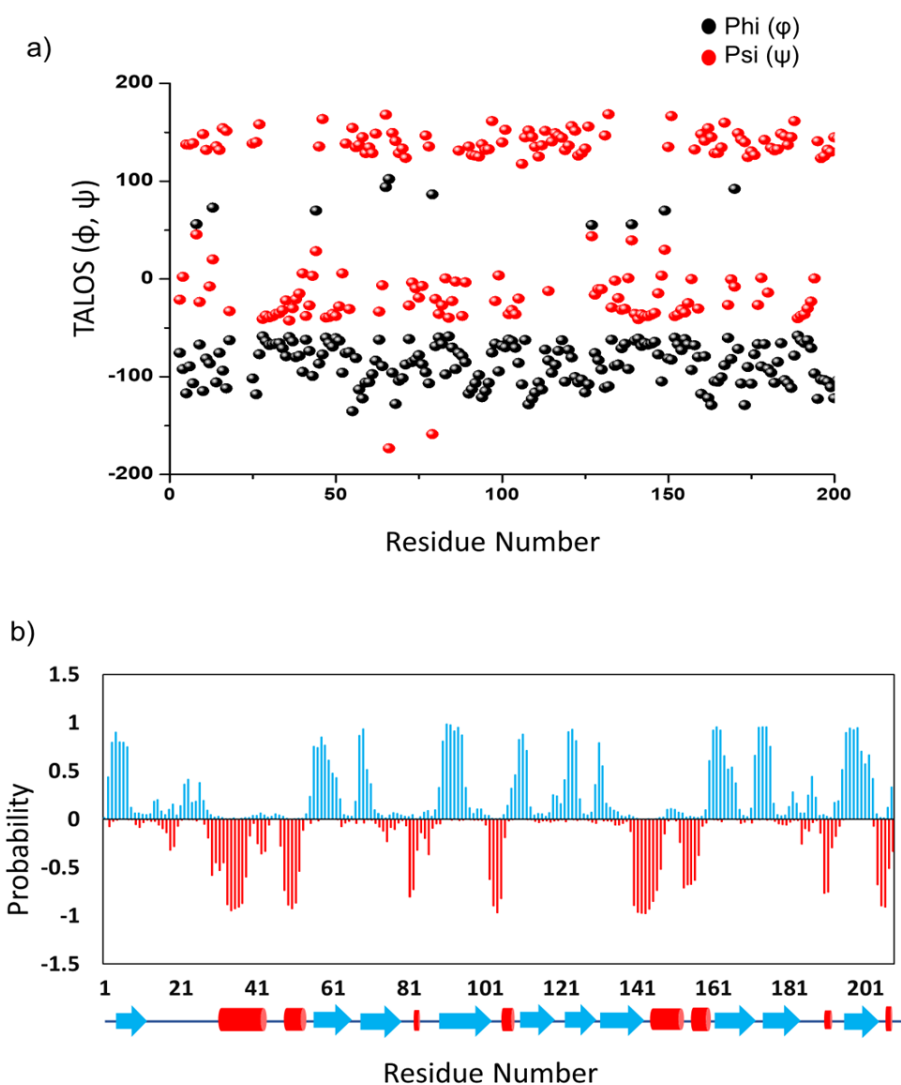


Figure 4.2 Summary of secondary structure prediction from TALOS-N webserver.

a) Backbone dihedral angles of Eap34 and b) Secondary structure probability of Eap34 as predicted by TALOS-N using NMR chemical shift derived from the backbone resonance assignments. β -strand probabilities are given by positive values, α -helices are given by negative values, and loop regions are given by values approximately from -0.5 to 0.5. The TALOS-N prediction of Eap34 with α -helices shown as cylinders and β -strands shown as arrows is presented at the bottom.

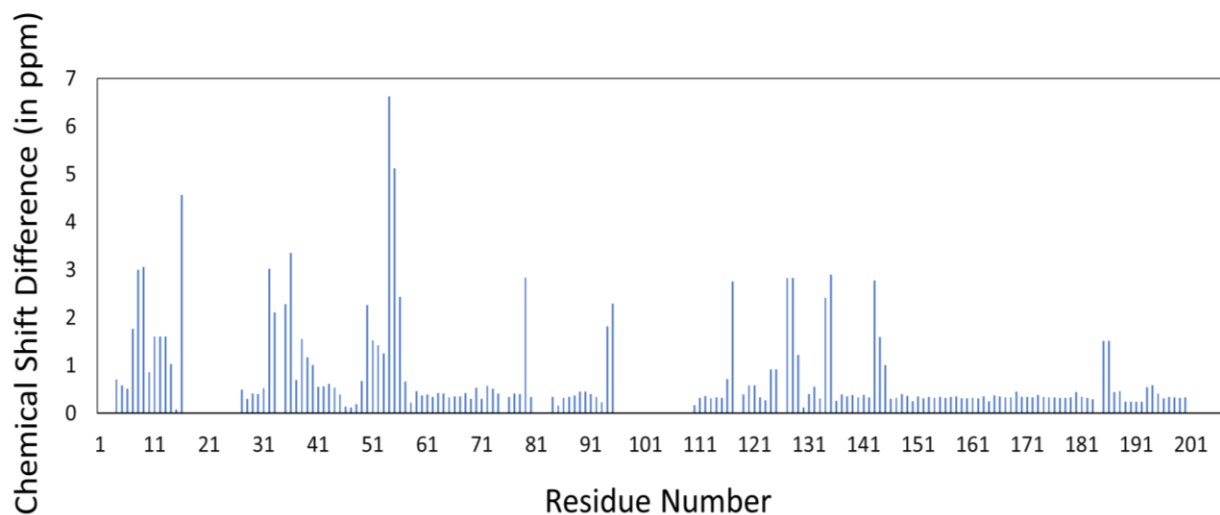


Figure 4.3 Bar plot of Chemical shift differences for domain 3 and domain 4 in Eap34, Eap3 and Eap4.

Table 4.1 Acquisition parameters of NMR experiments for backbone resonance assignments of Eap34.

Experiment Parameter	2D ^{15}N , ^1H HSQC		3D HNCB 3D HNCA(CO)			3D HNCA 3D HN(CO)CA			3D HNCACB		
	^1H	^{15}N	^1H	^{15}N	^{13}C	^1H	^{15}N	^{13}C	^1H	^{15}N	^{13}C
No. of Complex Points (Real + Imaginary)	2048	512	2048	96	64	2048	96	96	2048	96	96
Offset (ppm)	4.7	119.0	4.77	119.0	176.0	4.7	119.0	56	4.7	119.0	44.0
Spectral Width (ppm)	15.9	35	15.9	29.9	9.99	15.9	29.9	30	15.9	29.9	57.5
Inter-scan delay	1 sec										
No. of scans	8		8			8			16		

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Conflicts of interest/Competing interests

The authors declare no conflicts of interest.

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Chapter 5 - Complete non-proline backbone resonance assignments of the *S. aureus* neutrophil serine protease inhibitor, EapH1

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Abstract (174 words)

The *S. aureus* extracellular adherence protein (Eap) and its homologs, EapH1 and EapH2, serve roles in evasion of the human innate immune system. EapH1 binds with high-affinity and inhibits the neutrophil azurophilic granule proteases neutrophil elastase, cathepsin-G and proteinase-3. Previous structural studies using X-ray crystallography have shown that EapH1 binds to neutrophil elastase and cathepsin-G using a globally similar binding mode. However, whether the same holds true in solution is unknown and whether the inhibitor experiences dynamic changes following binding remains uncertain. To facilitate solution-phase structural and biochemical studies of EapH1 and its complexes with neutrophil granule proteases, we have characterized EapH1 by multidimensional NMR spectroscopy. Here we report a total of 100% of the non-proline backbone resonance assignments of EapH1 with BMRB accession number 50304.

Keywords

Backbone resonance assignment, Extracellular adherence protein, Protein NMR, *Staphylococcus aureus*

Biological context

Neutrophils are a primary innate cellular defense against many bacterial infections, including those caused by *Staphylococcus aureus* and related species (1, 2). Neutrophils contain several types of subcellular granules that are rich in antibacterial peptides, proteins, and enzymes capable of generating cytotoxic oxidants within the maturing phagosomal compartment (1–5). A major component of these granules are the neutrophil serine proteases (NSPs) neutrophil elastase (NE), cathepsin-G (CG), and proteinase-3 (PR3). These NSPs have been shown to directly attack certain bacterial cells and to digest bacterially-produced proteins, including many virulence factors from *S. aureus* (6–11). Consequently, NSPs are believed to be a cornerstone of neutrophils' anti-bacterial arsenal.

Like many successful pathogens, *S. aureus* has evolved mechanisms to evade detection and killing by the innate immune system. Among these are several secreted proteins that interfere with opsonization by complement fragments and subsequent phagocytosis by neutrophils, as well as killing within the neutrophil's phagosome (12–16). In 2014, Stapels and coworkers reported the identification of a family of three molecules known as EAP domain proteins that are potent, non-covalent, and selective inhibitors of NSPs (17, 18). Deletion of the genes encoding all three EAP domain proteins resulted in an approximately 100-fold reduction of bacterial burden in a mouse liver abscess model of *S. aureus* infection (17). This strongly suggests that inhibition of NSPs by EAP domain proteins plays an important role in *S. aureus* pathogenesis *in vivo*.

EAP domain proteins are characterized by an ~100 residue β -grasp motif that consists of a prominent α -helix superimposed diagonally across the face of anti-parallel β -sheet (19). The co-crystal structure of NE bound to the EAP domain protein, EapH1, showed that the inhibitor appears to completely occlude access to the protease active site, thereby explaining its potent effect on NE

activity (17). A subsequent structure of EapH1 bound to CG revealed a globally similar binding mode of the inhibitor relative to the protease active site (20). However, the CG/EapH1 interface involves mainchain-mainchain interactions to a much greater extent than the NE/EapH1 complex. Interestingly, the EapH1 molecule appears to experience changes in dynamics in certain regions in response to binding to its NSP targets. These include decreased levels of thermal motion in residues found at the NSP/EapH1 interface, but also increased motions in residues distal to the interface (17, 18, 21). This has raised questions regarding the importance of dynamic changes upon binding to EAP domain function, and whether these changes can be better characterized through use of biochemical and structural studies in solution.

Here we present the secondary structural features of *S. aureus* EapH1 in the free form in solution. After completing the ^1H , ^{15}N and ^{13}C NMR resonance assignment of EapH1, we are able to predict the secondary structure using the TALOS-N server using the observed chemical shifts. This work facilitates solution-phase structural and biochemical studies of EapH1 and its complexes with neutrophil granule proteases.

Methods and experiments

Protein expression and purification

A recombinant form of EapH1 from *S. aureus* strain Mu50 was overexpressed and purified from *E. coli* strain BL21(DE3). The general methodologies of Geisbrecht, Bouyain, Pop (2006) (22) and the modifications described by Herdendorf, Geisbrecht (2018) formed the basis for all expression and purification procedures, while all ^{13}C and ^{15}N isotopically-labeled samples were obtained through the protocols described by Woehl et al., 2016 (23). Briefly, following cell harvest and preparation of a soluble extract via microfluidization and high-speed centrifugation, EapH1

was isolated by immobilized metal ion affinity chromatography under native conditions at room temperature. Following initial purification, the affinity tag was removed by site-specific proteolysis with recombinant TEV protease, and the digested EapH1 was obtained by reapplying the sample to the metal ion affinity column and collecting the unbound fraction (24). Final purification was achieved using RESOURCE S cation exchange chromatography as described in Geisbrecht, Bouyain, Pop, 2006 (22). The purified EapH1 was dialyzed against ddH₂O and lyophilized for long-term storage prior to use. The mass and purity of the protein were verified using mass spectrometry (Ultra Flex III TOF, Bruker Daltonics) prior to acquisition of the NMR data to ensure the protein integrity.

NMR spectroscopy

A sample of recombinant EapH1 that had been isotopically labeled with ¹⁵N and ¹³C was used to collect all 3D NMR experiments. The final concentration of EapH1 was 0.6-1.0 mM prepared in sodium phosphate buffer (50 mM sodium phosphate, 50 mM NaCl, and 5 % (v/v) D₂O, pH 6.5) with DSS as an internal reference. All experiments were collected using an Agilent NMR instrument operating at ¹H Larmor frequency of 700 MHz equipped with an 5 mm HCN triple resonance cryogenically cooled probe with inverse detection and a Z-axis pulsed field gradient at 25 °C. 2D ¹H-¹⁵N HSQC experiments were used to optimize sample conditions. Along with 2D HSQC, a standard set of 3D NMR spectra were acquired for assigning the backbone resonances of EapH1 (parameters are listed in Table 5.1). All 3D NMR backbone resonance experiments were acquired with 12% of the Nyquist grid in the indirect dimensions using a non-uniform sampling. The grid points for sampling were chosen using a Poisson-Gap scheduler. Non-uniformly sampled spectra were reconstructed with an iterative soft thresholding (IST) method, hmsIST (25).

NMRpipe was used to process the uniformly sampled NMR spectra (26). After picking all the peaks in ^1H - ^{15}N HSQC spectrum, 3D HNCO and HN(CA)CO were used to pick the carbonyl resonances of EapH1 (27). The backbone resonance assignments were obtained using 3D HNCA, 3D HN(CO)CA and 3D HNCACB experiments (27, 28). Analysis of all spectra and resonance assignments were done using NMR spectrum analysis software CARA (<http://cara.nmr-software.org/portal/>) (29). The ^1H chemical shifts were referenced to the internal standard 2,2-dimethyl-2-silapentane-5-sulphonic acid (DSS) at 298K. However, ^{13}C and ^{15}N chemical shifts were referenced indirectly from DSS. The secondary structure of EapH1 was predicted using the TALOS-N platform (30).

Table 5.1 Parameters for all NMR spectra of EapH1 recorded on a 700 MHz NMR spectrometer at 298K

Experiment Parameter	2D ^{15}N , ^1H HSQC		3D HNCO 3D HNCA(CO)			3D HNCA 3D HN(CO)CA			3D HNCACB		
	^1H	^{15}N	^1H	^{15}N	^{13}C	^1H	^{15}N	^{13}C	^1H	^{15}N	^{13}C
No. of Complex Points (Real + Imaginary)	2048	512	2048	72*	128*	2048	72*	128*	2050	128*	128*
Offset (ppm)	4.7	117.08	4.77	117.08	176.04	4.7	117.08	55.07	4.77	117.08	45.08
Spectral Width (ppm)	15.95	33.99	15.9	29.9	9.99	15.95	33.99	30	15.95	33.99	63.99
Interscan delay	1 sec										
No. of scans	8		8			8			16		

* indicates the full grid points, however NUS was used to sample 12% of the full Nyquist grid.

Extent of assignments and data deposition

The 2D ^1H - ^{15}N HSQC spectrum of EapH1 is characterized by well-dispersed resonances (Figure 5.1). Amino acid numbering is based upon the EapH1 recombinant construct, beginning

with the extra “Gly-Ser-Thr” sequence at the N-terminus followed by the matured EapH1 sequence. 100% of the backbone ^1H and ^{15}N resonances from all 111 non-proline out of 114 total residues have been assigned using NMR experiments described in the methods and experiments section. Furthermore, 93% of the expected $^{13}\text{C}\beta$ resonances, 98% of $^{13}\text{C}\alpha$, and 100% of $^{13}\text{C}'$ from all non-proline residues have been unambiguously assigned based on a standard set of triple resonance spectra described above. The secondary structure elements of EapH1 were predicted by the TALOS-N program (30) using the resonance assignments of $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, $^{13}\text{C}'$, NH and ^1H (Figure 5.2). These results are consistent with a protein rich in β -strand structure, although residues 47-61 and 66-71 have strong α -helical propensity (Figure 5.2). The chemical shift assignments have been deposited in BioMagResBank (<http://www.bmrb.wisc.edu>) under the accession number 50304. The backbone resonance assignments along with secondary structural features of EapH1 will facilitate solution-phase structural and biochemical studies of this protein and its complexes with neutrophil azurophilic granule proteases.

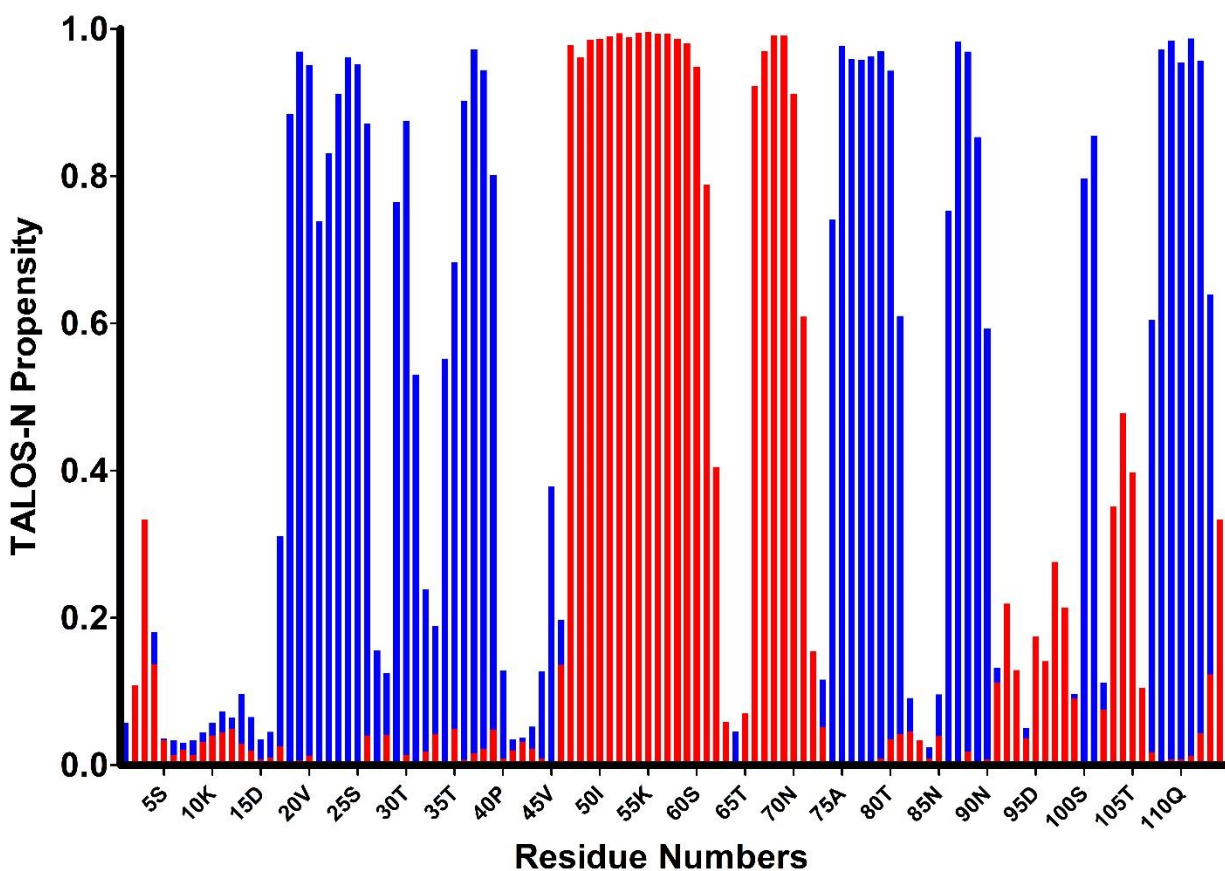


Figure 5.2 TALOS-N prediction of secondary structure propensity for EapH1 based on chemical shift values of $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and $^{13}\text{C}'$, NH and ^1H .

Blue bars depict β -sheets while red bars indicate α -helical properties.

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The authors declare no conflict of interest.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: We give our consent to publish this manuscript in BNA journal.

Availability of data and material: NMR data of EapH1 has been deposited to BMRB accession number 50304.

Competing interests: The authors declare no conflict of interest.

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Chapter 6 - Simultaneous inhibition of two neutrophil serine proteases by the *S. aureus* innate immune evasion protein EapH2

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Running title: Dual inhibition of neutrophil serine proteases by EapH2

Abstract (250 words)

Extracellular adherence protein domain (EAPs) proteins are high affinity, selective inhibitors of neutrophil serine proteases (NSP), including cathepsin-G (CG) and neutrophil elastase (NE). Most *S. aureus* isolates encode for two EAPs, EapH1 and EapH2, that contain a single functional domain and share 43% identity with one another. Although structure/function investigations from our group have shown that EapH1 uses a globally similar binding mode to inhibit CG and NE, NSP inhibition by EapH2 is incompletely understood due to a lack of NSP/EapH2 cocrystal structures. To address this limitation, we further studied NSP inhibition by EapH2 in comparison with EapH1. Like its effects on NE, we found that EapH2 is a reversible, time-dependent, and low nanomolar affinity inhibitor of CG. We characterized an EapH2 mutant which suggested that the CG binding mode of EapH2 is comparable to EapH1. To test this directly, we used NMR chemical shift perturbation to study EapH1 and EapH2 binding to CG and NE in solution. Although we found that overlapping regions of EapH1 and EapH2 were involved in CG binding, we found that altogether distinct regions of EapH1 and EapH2 experienced changes upon binding to NE. An important implication of this observation is that EapH2 might be capable of binding and inhibiting CG and NE simultaneously. We confirmed this unexpected feature by solving crystal structures of the CG/EapH2/NE complex and demonstrating their functional relevance through enzyme inhibition assays. Together, our work defines a new mechanism of simultaneous inhibition of two serine proteases by a single EAP protein.

Introduction

Extracellular adherence protein domain (EAPs) proteins are a family of three high-affinity non-covalent serine protease inhibitors expressed and secreted by the pathogenic bacterium *Staphylococcus aureus* as part of its innate immune evasion repertoire (1-3). EAPs exhibit a high degree of selectivity for the serine proteases found in the azurophilic granules of neutrophils (3-5), including cathepsin-G (CG) and neutrophil elastase (NE), and fail to inhibit the activity of closely related serine proteases such as plasmin, kallikrein, and thrombin (3). Unlike many other *S. aureus* immune evasion proteins (6, 7), EAPs bind and inhibit murine NSPs in addition to their human orthologs (3). Consistent with this observation, Stapels et al. showed that concurrent deletion of the genes encoding all three EAPs results in a statistically significant, ~100-fold reduction of *S. aureus* burden in a mouse liver abscess infection model (3). These results strongly suggest that NSP inhibition by EAPs is a contributing factor to *S. aureus* pathogenesis *in vivo*.

S. aureus strains typically produce three unique EAPs, which are characterized by an ~100 residue β -grasp domain that defines this protein family (8-10). Two of these proteins, known as EapH1 and EapH2, are comprised of a single EAP repeat (Fig. 6.1A), whereas the eponymous Extracellular Adherence Protein (Eap) exists in four, five, or six domain-containing isoforms depending on the *S. aureus* strain examined (8-10). Although the multidomain Eap protein has been assigned numerous functions, including those of a broad-spectrum adhesin that binds to various host extracellular matrix proteins (11) and inhibition of the classical and lectin pathways of complement (12), single EAP domains in isolation are sufficient to potently inhibit NSPs (3). Since EAP domains are not related in sequence or structure to other classes of serine protease inhibitors (3), and since they exhibit an unusual time-dependent inhibitory character (13-15), we have been taking a systematic approach to understand the structure/function relationships and

mechanisms that underlie their effects on NSPs. In these efforts, EapH1 has served as a paradigm since we succeeded in crystallizing this inhibitor initially bound to NE (3) and thereafter to CG (15) (Fig. S1A-B). EapH2 has been refractory to crystallization with either of these NSPs, however, which has led to significant limitations in our understanding of this protein.

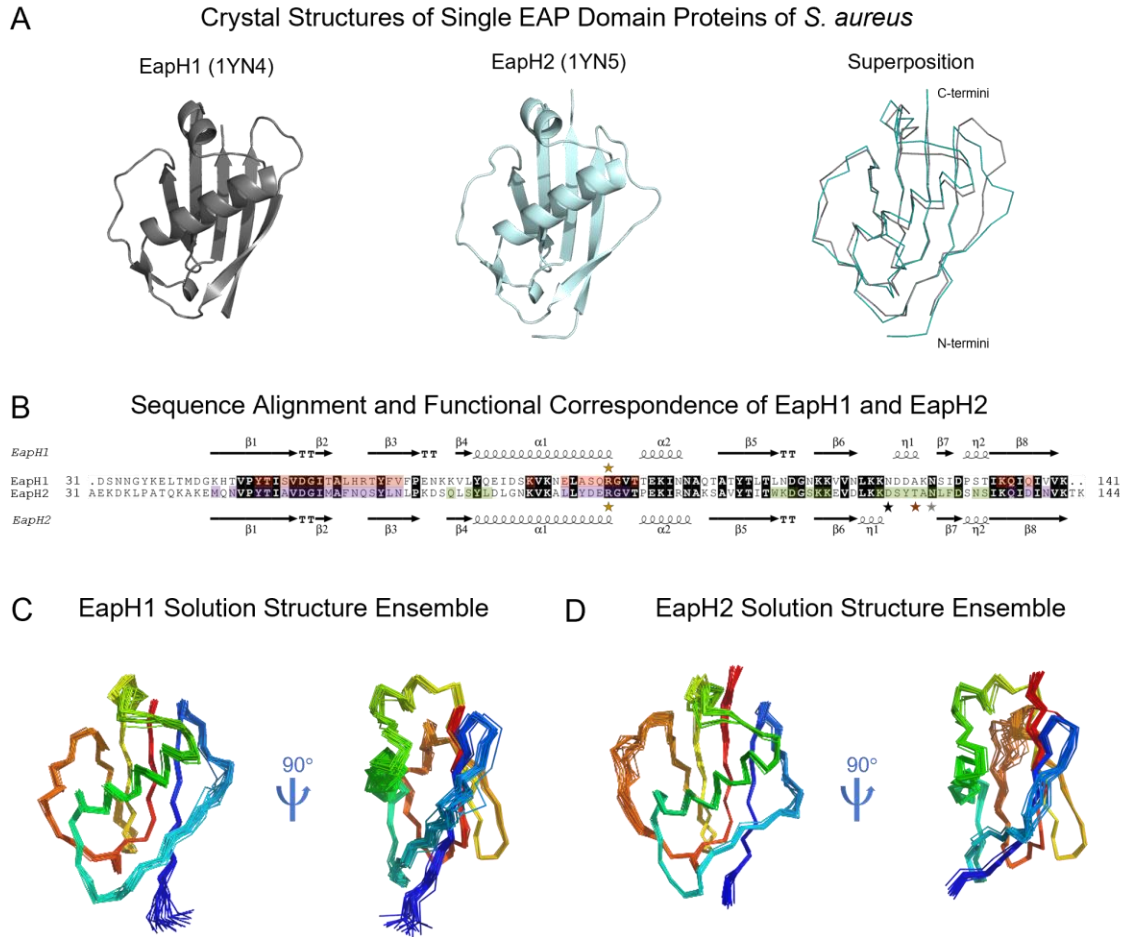


Figure 6.1 Sequence and structural relationships between EapH1 and EapH2.

The genome of *S. aureus* strain Mu50 encodes three EAP proteins known as the Extracellular Adherence Protein (Eap) (NCBI reference sequence WP_001557458), EapH1 (NCBI reference sequence WP_000769705), and EapH2 (NCBI reference sequence WP_000769694). (A) Crystal structures for EapH1 (1.8 Å resolution, PDB code 1YN4) and EapH2 (2.2 Å resolution, PDB code 1YN5) have been previously reported (8). Representative models for each protein superimpose with an R.M.S.D. of 0.60 Å for the 82 of 97 C α positions that lie within a 5.0 Å distance threshold.

(B) The sequences of the matured polypeptides for EapH1 (top) and EapH2 (bottom) were aligned with Clustal Omega (52) and displayed along with their secondary structure elements using EsPript (51). Residue numbers from the reference sequences above are shown at the left and right of each row. EapH1 and EapH2 share 43% sequence identity and the conserved positions are shown in reverse typeface. Positions in EapH1 found at the interface in cocrystal structures with CG (15) and NE (3) are highlighted in orange. Positions in EapH2 found at the CG interface are highlighted purple while positions found at the NE interface are highlighted green, as inferred from the CG/EapH2/NE ternary complex structures reported here (*please see Fig. 6.4, Fig. S1, & Fig. S5*). The locations of functionally important residues established by mutagenesis are indicated as follows: EapH1-R89/EapH2-R90 (gold star), EapH2-N127 (silver star), EapH2-T125 (bronze star), and EapH2-D122 (black star). (C) Orthogonal views of the solution structure ensemble for EapH1 are shown. Protein models are depicted by their C α positions with the N-terminus in blue and C-terminus in red. (D) Orthogonal views of the solution structure ensemble for EapH2 are shown. Protein models are depicted by their C α positions with the N-terminus in blue and C-terminus in red.

Because the sequences of CG and NE are only 34% identical to one another (2, 15), we expected to find fundamentally different binding modes for EapH1 with these two NSPs (15). However, when we compared the CG/EapH1 and NE/EapH1 crystal structures, we immediately noticed that EapH1 employed a globally similar binding mode to inhibit both proteases (15). Examination of the EapH1 residues found at the interface with CG and NE revealed that the NSP-binding sites overlap, and that they are comprised primarily of amino acids from the β 1- β 3 region as well as from the C-terminal half of α 1 of the inhibitor (Fig. 6.1B). A conserved arginine (i.e. EapH1-R89 and EapH2-R90) lies at the C-terminus of α 1 in EapH1, EapH2, as well as other EAP proteins (8). Consistent with the CG/EapH1 and NE/EapH1 crystal structures, we found that this arginine is a major contributor to EapH1 function, since the EapH1-R89M mutant is roughly 100-fold diminished in its inhibition of CG (15) and more than 15,000-fold diminished in its inhibition of NE (13). However, when we characterized the corresponding EapH2-R90M mutant, we unexpectedly found wild-type inhibitory activity against NE (c.f. $K_{I,WT} = 2.1$ nM vs $K_{I,R90M} = 2.3$ nM) (14).

EapH1 and EapH2 are 43% identical in sequence and their crystal structures are essentially indistinguishable from one another (Fig. 6.1A-B) (8). Similarly, the solution structure ensembles of EapH1 and EapH2 determined by multidimensional solution NMR spectroscopy retain all fundamental features seen in the earlier crystal structures (Fig. 6.1C-D & Table 6.1). This high level of sequence/structure identity, coupled with the inability to crystallize EapH2 with either NSP, presented challenges to explaining the differences in NE inhibition between EapH1 and EapH2. To circumvent this issue, we used a computational approach to generate a model for the NE/EapH2 complex (16). Surprisingly, this model suggested that a distinct region of EapH2 extending from the C-terminus of $\beta 5$ to the beginning of $\beta 8$ might be involved in inhibiting NE (Fig. 6.1B & Fig. S1C). When we tested the accuracy of this model by site-directed mutagenesis, we found that two residues predicted to interface with NE contributed to EapH2 function (Fig. 6.1B), as the EapH2-T125A and EapH2-N127A mutants lack time-dependent character and are ~25- and ~200-fold diminished in their inhibition of NE (14). Nevertheless, the absence of empirical structural data makes it difficult to fully appreciate the roles of these residues, or to understand whether they might contribute to CG inhibition by EapH2.

Table 6.1 Summary of statistics for NMR solution structure ensembles of EapH1 and EapH2

PDB Accession Code	EapH1 ^a 8GDH	EapH2 ^b 8GDG
NOE-based distance restraints		
Intra-residue ($i = j$)	269	321
Sequential ($ i-j = 1$)	434	502
medium range ($1 < i-j < 5$)	276	239
Long Range ($ i-j \geq 5$)	253	251
Total	1232	1313
Hydrogen bond distance restraints	56	78
Dihedral angle restraints (Φ and Ψ)	183	189
Restraint Violations		
NOE > 0.2 Å	0.75	8.8
Dihedral angle $> 5^\circ$	0	0
RMSD from the mean structure (Å) ^c		
Main chain	0.69 ± 0.11	0.68 ± 0.24
Heavy atoms	1.21 ± 0.13	1.24 ± 0.26
RMSD from experimental restraints		
NOE-based distance restraints (Å)	0.012 ± 0.001	0.014 ± 0.001
Dihedral angle restraints ($^\circ$)	0.355 ± 0.057	0.404 ± 0.035
Deviations from ideal geometry		
RMS deviation for bond angles ($^\circ$)	0.2	0.2
RMS deviation for bond lengths (Å)	0.001	0.001
Ramachandran analysis (PROCHECK-NMR)		
Most favored regions (%)	90.7	85.3
Additionally allowed regions (%)	9.3	14.5
Generously allowed regions (%)	0	0.1
Residues in disallowed regions	0	0.1
Protein Structure Validation Suite Analyses (version 1.5) ^d		
PROCHECK G-factor (all dihedral)	-0.78 N/A (-4.61)	-0.79 N/A (-4.67)
PROCHECK G-factor (phi/psi)	-0.39 N/A (-1.22)	-0.45 N/A (-1.46)
Verify3D	0.24 ± 0.02 (-3.53)	0.17 ± 0.02 (-4.65)
ProsaII (-ve)	0.56 ± 0.03 (-0.37)	0.56 ± 0.04 (-0.37)
MolProbity clashscore ^e	13.68 ± 4.16 (-0.82)	9.23 ± 2.85 (-0.06)
MolProbity clashcore Analysis (version 4.5.1) ^f	22.95	16.16

^aStatistics calculated for 99 ordered residues of the EapH1 ensemble corresponding to positions 42-141 of the reference sequence.^bStatistics calculated for 100 ordered residues of the EapH2 ensemble corresponding to positions 44-144 of the reference sequence.^cCalculated using Cyana 2.1.^dValues in parentheses represent Z-scores.^eClashscore obtained from the PSVS server.^fClashscore obtained from the Molprobity server.

Here we investigated the structural and functional basis for inhibition of CG and NE by EapH2 in comparison to EapH1. As with EapH1, we found that EapH2 is a time-dependent inhibitor of CG and that the conserved arginine residue (i.e. EapH2-R90) plays an important role in this function. Using solution NMR chemical shift perturbation experiments, we found that EapH2 residues corresponding to the CG and NE interface in EapH1 also experience changes upon binding to CG, but that residues on the opposing face of EapH2 undergo changes upon binding to NE. This raised the possibility that EapH2 might be capable of binding to CG and NE concurrently, which we

confirmed through crystallization of a CG/EapH2/NE ternary complex. Finally, we provide evidence that EapH2, but not EapH1, can inhibit both CG and NE simultaneously. To our knowledge, this is the first report describing simultaneous inhibition of two proteases by a molecule that contains only a single functional domain.

Results

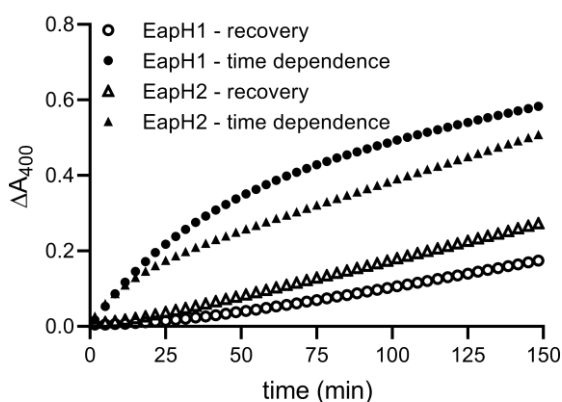
EapH2 is a Reversible, Time-Dependent Inhibitor of CG

We have previously shown that EapH1 is a reversible, time-dependent inhibitor of CG and NE (13, 15). Although we have reported similar properties for inhibition of NE by EapH2 (14), whether the same holds true for inhibition of CG remains unclear. To test whether EapH2 inhibition of CG is reversible, we preincubated CG with EapH2 for 30 mins, diluted the sample with buffer containing a CG substrate, SA₂PF-pNA, and followed the reaction for 150 mins. We observed a lag in reaction progress that gradually gave way to a linear, steady-state reaction rate (Fig. 6.2A), indicating the presence of free CG in the assay. However, when we exposed free CG simultaneously to substrate and EapH2, we observed reaction kinetics with an initial phase comparable to the uninhibited reaction, but which eventually slowed to a steady-state inhibited rate (Fig. 6.2A). These results are diagnostic of time-dependent inhibition (13-15).

Since initial velocity measurements cannot be used to determine the apparent inhibition constant (i.e. K_i) of time-dependent inhibitors, we utilized progress curve analysis to evaluate CG inhibition by EapH2 (17,18). In this approach, reactions are followed across longer time regimes where substrate depletion and product accumulation can be accounted for during data analysis (19,20). We collected CG progress curves using two enzyme concentrations, three substrate concentrations, and five different EapH2 concentrations. We then fit the data to a rapid-equilibrium competitive

inhibition model to determine the on-rates and off-rates of EapH2 for CG, and thereafter the overall inhibition constant, K_I (please see Experimental Procedures). Using this strategy, we found that EapH2 ($K_I=25.4$ nM) is an ~ 1.7 -fold more potent inhibitor of CG than EapH1 (Table 6.2) (15). Although the on-rate of EapH2 (i.e. k_4) for CG (2.4×10^4 M $^{-1}$ s $^{-1}$) is ~ 3 -fold faster than that of EapH1 (8.4×10^3 M $^{-1}$ s $^{-1}$), the off-rate of EapH2 (i.e. k_5) for CG (6.0×10^{-4} s $^{-1}$) is ~ 1.6 -fold faster than that of EapH1 (3.7×10^{-4} s $^{-1}$) as well. We noted that inhibitory potency of EapH2 for CG is roughly an order of magnitude weaker than its potency against NE ($K_I=2.1$ nM) (14). This stands in contrast to EapH1, which inhibits CG ($K_I=43.7$ nM) over $\sim 2,000$ -fold more weakly than it inhibits NE ($K_I=21$ pM) (13).

A Inhibition of CG by EapH1 and EapH2



Inhibition of CG by EapH2
Representative Progress Curves

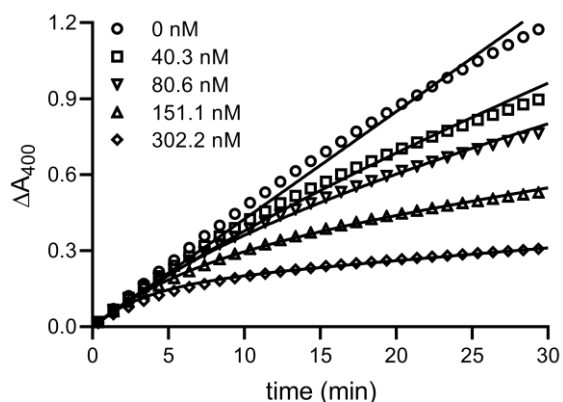


Figure 6.2 EapH2 is a reversible, time-dependent inhibitor of CG.

Cleavage of the chromogenic substrate SA₂PF-pNA by CG was followed spectrophotometrically in presence of inhibitors. (A) Progress curves resulting from exposure of 10.2 nM CG to an assay mixture containing 1.14 mM SA₂PF-pNA and 154 nM EapH1 (filled circle) or 154 nM EapH2 (filled triangle), as well as curves resulting from dilution of preincubated samples of CG/EapH1 (open circle) or CG/EapH2 (open triangle) with an assay mixture that gave identical final concentrations to the previous experiment. (B) Representative fitted progress curves for inhibition of CG by EapH2. Assay mixtures consisted of 16.3 nM CG, 5.6 mM SA₂PF-pNA and the EapH2 concentrations noted in the figure inset.

We have also shown that EapH1-R89 is an important functional determinant to inhibition of CG and NE (13, 15). Although these observations are consistent with crystal structures of

EapH1 bound to CG and NE (3, 15), the lack of a CG bound structure for EapH2 raised questions about the nature of the CG-binding mode for this inhibitor. To gain initial insight into this question, we characterized CG activity in the presence of the EapH2-R90M mutant. Interestingly, we found that EapH2-R90M inhibits CG in a time-dependent manner but with a potency ~ 17.8 weaker than wild-type EapH2 ($K_I=454$ nM) (Table 6.2). This loss of function is due to both a decreased on-rate (~ 2.75 -fold) and an increased off-rate (~ 6.5 -fold) of the mutant. Considering the location of EapH2-R90 (Fig. 6.1B), this result provided evidence that EapH2 might inhibit CG through an EapH1-like binding mode. Furthermore, since the same mutant has no effect on NE inhibition by EapH2 (14), we hypothesized that EapH2 uses distinct binding modes to inhibit CG and NE.

Table 6.2 Rate constant estimates of NSP inhibition determined by progress curve analysis

Inhibition of CG ^a										
Inhibitor	<i>k</i> ₁ (M ⁻¹ s ⁻¹)	SD	<i>k</i> ₃ (s ⁻¹)	SD	<i>k</i> ₄ (M ⁻¹ s ⁻¹)	SD	<i>k</i> ₅ (s ⁻¹)	SD	K _i (nM)	SD
EapH1 WT ^b	3.1x10 ³	1.5x10 ²	8.1	1.3	8.4x10 ³	4.0x10 ²	3.7x10 ⁻⁴	8.3x10 ⁻⁵	43.7	8.6
EapH1 R89M	3.0x10 ³	1.6x10 ³	10.2	3.1	1.1x10 ³	1.8x10 ²	1.6x10 ⁻³	1.9x10 ⁻⁴	1446	202
EapH2 WT	3.7x10 ³	1.3x10 ³	10.8	3.7	2.4x10 ⁴	3.8x10 ³	6.0x10 ⁻⁴	4.7x10 ⁻⁵	25.4	2.6
EapH2 R90M	3.2x10 ³	5.7x10 ²	10.4	2.2	8.7x10 ³	5.5x10 ²	3.9x10 ⁻³	1.5x10 ⁻⁴	454	26
Inhibition of NE ^c										
Inhibitor	<i>k</i> ₁ (M ⁻¹ s ⁻¹)	SD	<i>k</i> ₃ (s ⁻¹)	SD	<i>k</i> ₄ (M ⁻¹ s ⁻¹)	SD	<i>k</i> ₅ (s ⁻¹)	SD	K _i (nM)	SD
EapH2 WT ^d	3.1x10 ⁵	4.5x10 ⁴	7.8	0.2	2.9x10 ⁵	4.3x10 ⁴	5.9x10 ⁻⁴	2.1x10 ⁻⁵	2.1	0.3
EapH2 D122A ^d	6.9x10 ⁵	5.0x10 ⁵	8.3	2.3	1.2x10 ⁵	1.5x10 ⁴	3.1x10 ⁻³	1.2x10 ⁻⁴	26	3
EapH2 D130A	3.6x10 ⁵	1.8x10 ⁵	5.6	0.4	3.4x10 ⁵	4.2x10 ⁴	8.3x10 ⁻⁴	9.3x10 ⁻⁵	2.5	0.1
EapH2 D122A/D130A	1.4x10 ⁶	1.4x10 ⁶	5.9	0.8	2.5x10 ⁵	2.1x10 ⁴	5.4x10 ⁻³	1.4x10 ⁻³	22	3

^aRate constant estimates are the average of at least three individual sets of 30 progress curves (two [CG], three [substrate] per [CG], and five [inhibitor] per [substrate]).

^bValues for EapH1 and the EapH1-R89M mutant differ slightly from those reported previously due to modifications in the CG assay procedure (15).

^cRate constant estimates are the average of at least three individual sets of 45 progress curves (three [NE], three [substrate] per [NE], and five [inhibitor] per [substrate]).

^dValues for EapH2 and the EapH2-D122A mutant have been previously reported by Herdendorf & Geisbrecht (14).

NMR Chemical Shift Perturbation Studies on EapH2 Identify Distinct Binding Sites for CG and NE

To better understand how EapH1 and EapH2 bind NSPs in solution, we conducted NMR titration studies on isotopically labeled inhibitors and natural abundance CG and NE that was purified from human-derived sources and obtained commercially. Using backbone assignments

for EapH1 (BMRB accession number 50304; (21)) and EapH2 (BMRB accession number 27540; (22)), we monitored changes in 2D ^1H - ^{15}N HSQC spectra collected on a fixed concentration of $^{15}\text{N}/^{13}\text{C}$ -labeled inhibitor (i.e. 100 mM) either in the absence (Fig. S2) or presence of increasing concentrations of CG or NE; since the concentration of labeled EapH1 and EapH2 we used was far above the K_D/K_I values for the various complexes under investigation (Table 6.2; (13-15)), the titration series approximated a linear increase in concentration of the bound complex with no free CG or NE present. Residues involved in protein-protein interactions exhibit altered chemical shift properties (23). Therefore, by superimposing spectral series for titrations with CG (Fig. S3) or NE (Fig. S4), we identified residues in EapH1 and EapH2 whose resonances displayed either changing peak intensities or chemical shifts. We then calculated the chemical shift perturbation index for each EapH1 or EapH2 residue in the presence of CG or NE (*please see Experimental Procedures*). Finally, we plotted these results and mapped the residues with CSP values above 0.1 on the structures of EapH1 and EapH2 to characterize the respective NSP/inhibitor interfaces.

As a control, we first examined the titration series for CG binding to EapH1 since a crystal structure for the CG/EapH1 complex has been reported previously (Fig. S1A; (15)). These experiments show that residues EapH1-D42 through EapH1-F66 undergo the largest chemical shift changes in the ^1H - ^{15}N HSQC spectra (Fig. S3A) and display the largest CSP indices (Fig. 6.3A). As these residues comprise b1-b3 of EapH1 (Fig. 6.1B), these observations obtained from solution state information are consistent with the protein-protein interface seen in the CG/EapH1 crystal structure. We then examined the corresponding titration series for CG binding to EapH2 since there is no crystal structure available for this complex. These experiments show that residues EapH2-V48 through EapH2-L67 and EapH2-D88 through EapH2-G91 undergo the largest chemical shift changes (Fig. S3B) and display the largest CSP indices (Fig. 6.3B). As with EapH1,

these residues comprise b1-b3 of EapH2 (Fig. 6.1B). They also include EapH2-R90, which we identified earlier as being an important determinant of CG inhibition by EapH2 (Table 6.2). Together, these observations strongly suggest that EapH2 inhibits CG through an EapH1-like binding mode.

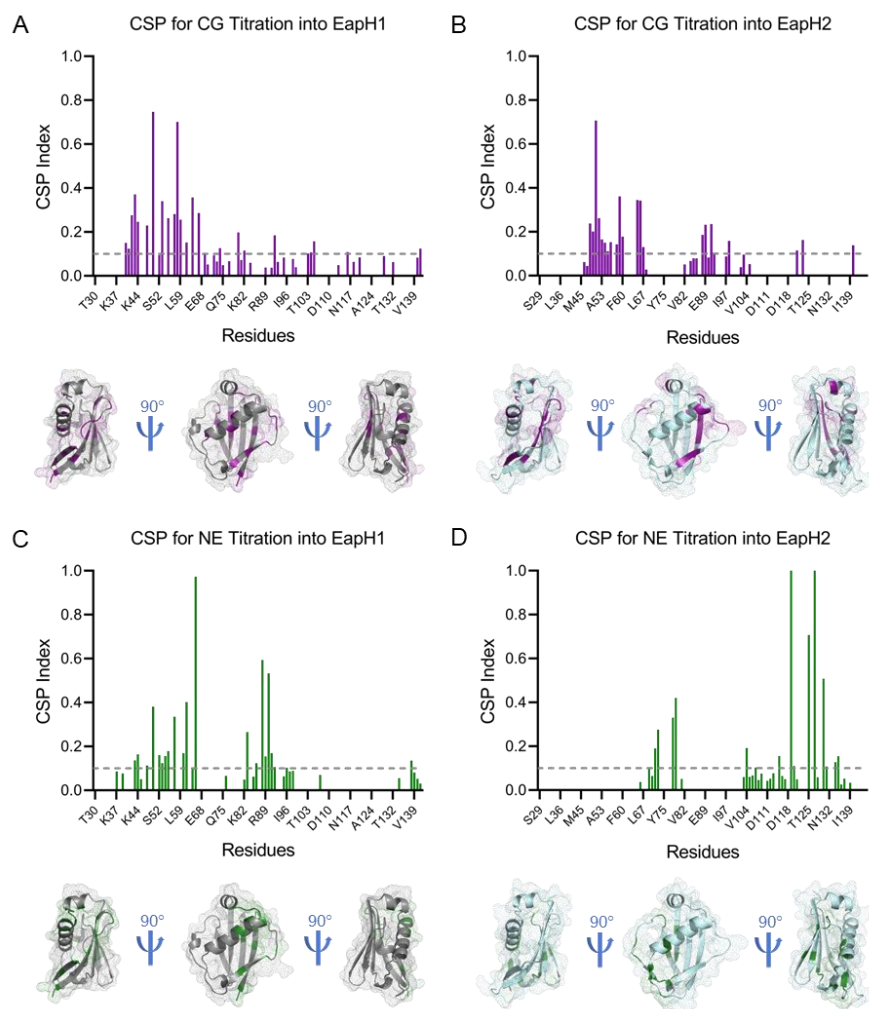


Figure 6.3 Mapping of CG and NE interaction sites on EapH1 and EapH2 by chemical shift perturbation.

Isotopically enriched forms of EapH1 and EapH2 were prepared and exposed to increasing concentrations of CG and NE. ^1H - ^{15}N HSQC spectra were collected and analyzed to map protein-protein interaction sites by chemical shift perturbation (*please see Experimental Procedures*). (A) Observed CSP index for EapH1 residues following titration with CG. The images at the bottom of the panel show three orthogonal views of EapH1 where the residues with CSP values greater than 0.1 are colored purple. (B) Observed CSP index for EapH2 residues following titration with CG.

The images at the bottom of the panel show three orthogonal views of EapH2 where the residues with CSP values greater than 0.1 are colored purple. (C) Observed CSP index for EapH1 residues following titration with NE. The images at the bottom of the panel show three orthogonal views of EapH1 where the residues with CSP values greater than 0.1 are colored green. (D) Observed CSP index for EapH2 residues following titration with NE. The images at the bottom of the panel show three orthogonal views of EapH2 where the residues with CSP values greater than 0.1 are colored green. Please note that all residue numbers in this Figure correspond to the positions in the reference sequences as given in Fig. 6.1 whereas the numbers in Fig. S2-4 correspond to the sequences of the recombinant proteins following cleavage by TEV protease.

A crystal structure of the NE/EapH1 complex shows globally similar binding pose for the inhibitor when compared to a crystal structure of CG/EapH1 (Fig. S1A-B; (3, 15)). Therefore, we examined the titration series for NE binding to EapH1 to determine if the same was true in solution. These experiments show that residues EapH1-G43 through EapH1-V65 and EapH1-N83 through EapH1-T92 experience the greatest chemical shift changes in the ^1H - ^{15}N HSQC spectra (Fig. S4A) and display the largest CSP indices (Fig. 6.3C). These residues comprise b1-b3 of EapH1 as well as the C-terminal half of a1 (Fig. 6.1B). This latter region also includes EapH1-R89, which we previously identified as a critical position for NE inhibition by EapH1 (13). Since these NMR titration data correspond well to the crystal structure of NE/EapH1, we examined the titration series for NE binding to EapH2 to obtain solution structural information on this complex. These experiments show that residues EapH2-Q72 through EapH2-G79 as well as EapH2-L119 through EapH2-D130 undergo the greatest chemical shift changes (Fig. S4B) and display the largest CSP indices (Fig. 6.3D). Significantly, these residues lie within the short b4 of EapH2 and the N terminal-most segment of a1, as well the region that connects b6-b8 (Fig. 6.1B). This latter region encompasses EapH2-D122, EapH2-T125, and EapH2-N127, which we identified previously as contributing to NE inhibition by EapH2 (14). Consequently, these observations establish that a distinct region of EapH2 is involved in its inhibition of NE when compared to CG, and further differentiate EapH2 from its closest homolog, EapH1.

Crystal Structure of the CG/EapH2/NE Complex Reveals a New Mode of Protease Inhibition

The NMR studies described above raised the intriguing possibility that EapH2 might be capable of binding CG and NE simultaneously, and thereby forming a ternary CG/EapH2/NE complex containing two NSPs bound to a single inhibitor. To explore this possibility, we prepared samples containing equal concentrations of CG, EapH2, and NE and used them to carry out high throughput sitting drop crystallization screening. We identified numerous conditions containing moderate amounts of salts (0.2 M) and polyethylene glycol-3350 (25% (w/v)) across mildly acidic and basic ($5.5 < \text{pH} < 8.5$) pH values that resulted in crystallization (*please see Experimental Procedures*). Following optimization, we collected X-ray diffraction data to 1.8 Å limiting resolution on a crystal grown at pH 5.5 and solved the structure by molecular replacement using structures of the individual proteins as search models. We refined this structure of the CG/EapH2/NE ternary complex to R_{work} and R_{free} values of 21.2 and 24.9%, respectively (Table 6.3).

Table 6.3 X-ray diffraction data, structure solution, and refinement statistics^a

PDB Accession Code	CG/EapH2/NE, pH5.5 8G24	CG/EapH2/NE, pH 7.5 8G25	CG/EapH2/NE, pH 8.5 8G26
<i>Data Collection</i>			
Space Group	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Wavelength (Å)	1.000	1.000	1.000
Cell Dimensions			
a, b, c (Å)	49.07, 68.60, 82.42	86.15, 68.43, 141.90	49.46, 68.92, 82.52
α , β , γ (°)	102.52	102.76	102.38
Resolution (Å)	50.00-1.82	50.00-1.80	50.00-1.85
Wilson B-Factor (Å ²)	31.8	25.4	28.9
Completeness (%)	98.3 (98.9)	98.5 (98.6)	99.1 (99.8)
I / σ I	10.9 (1.4)	10.6 (1.3)	10.2 (1.9)
R _{pit}	0.057 (0.577)	0.075 (0.588)	0.063 (0.657)
CC _{1/2}	0.985 (0.466)	0.979 (0.617)	0.984 (0.560)
Redundancy	6.5 (5.6)	6.5 (5.6)	6.5 (5.9)
<i>Refinement</i>			
Resolution (Å)	47.90-1.82	46.13-1.80	39.56-1.85
Number of Reflections	44,822	133,623	43,792
R _{work} / R _{free} (%)	21.2 / 24.9	24.8 / 28.3	20.3 / 23.8
Atoms Modeled			
Total	8,729	13,335	8,810
Amino Acid Residues	544	1,632	544
Water	163	633	219
Ramachandran Plot			
Favored/Allowed (%)	94.05 / 5.76	96.10 / 3.71	97.03 / 2.60
Average B-Factors (Å ²)	77.0	50.0	60.0
R.M.S. Deviations			
Bond Lengths (Å)	0.005	0.005	0.005
Bond Angles (°)	0.82	0.72	0.78

^a Values in parentheses are for the highest resolution shell.

The structure of the CG/EapH2/NE ternary complex consists of EapH2 with CG and NE bound at the sites predicted by our earlier NMR studies (Fig. 6.4). The CG/EapH2 interface employs the same global binding mode that we described for EapH1 in both its CG- and NE-bound states (Fig. S1A-B,D) (3, 15). By contrast, the NE/EapH2 interface employs an alternative binding mode involving largely the same region of EapH2 predicted in our earlier computational model (16), with the exception that EapH2 is flipped in the orientation of its β -sheet relative to the NE active site (Fig. S1C,E). This binding mode is also equivalent to that we recently reported for the first and second domains of *S. aureus* Eap in their NE-bound forms (24). When compared to the crystal structure of unbound EapH2 (Fig. 6.1A) (8), EapH2 appears to undergo minor structural rearrangements in both protease-binding sites to facilitate simultaneous interaction with CG and NE. However, when the NMR solution structure ensemble is considered (Fig. 6.1D), these changes

seem to be accommodated by the inherent structural flexibility within the EapH2 protease-binding sites.

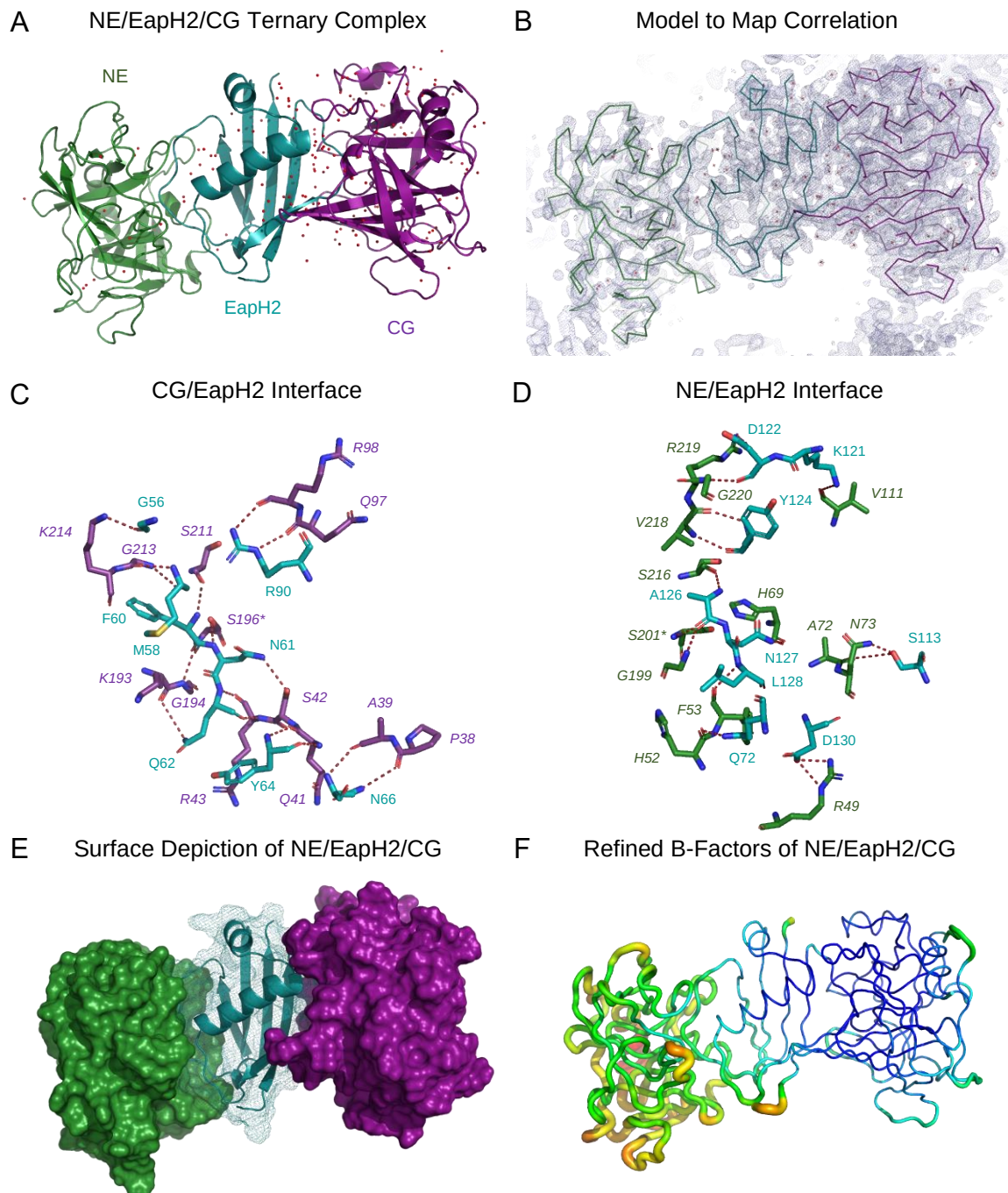


Figure 6.4 Crystal structure of EapH2 bound to CG and NE defines a physical basis for simultaneous inhibition of NSPs.

The ternary complex of CG/EapH2/NE was crystallized, and its structure was solved by molecular replacement methods. (A) Final model of the asymmetric unit for CG/EapH2/NE crystallized at pH 5.5 and refined at 1.82 Å limiting resolution. CG (purple), EapH2 (teal), and NE (green) are depicted as ribbon diagrams, while ordered solvent molecules are shown as red asters. The N-terminus of EapH2 is positioned centrally at the bottom of the protein as drawn. (B) Model-to-map correlation for the CG/EapH2/NE structure shown in an identical orientation to Panel A. Proteins are depicted as wire traces where only their C α positions are shown. The 1.82 Å resolution 2Fo-Fc electron density map contoured at 1.2 σ is drawn as a grey mesh. (C) Schematic view of interactions near the CG active site as inhibited by EapH2. Residues of interest are drawn in ball-and-stick convention with proposed hydrogen bonds as dashed lines. Residues from CG (purple) are labeled with italicized letters, while residues from EapH2 (teal) are labeled with normal typeface. The position of the CG catalytic serine is highlighted with an asterisk (i.e. *S196**). (D) Schematic view of interactions near the NE active site as inhibited by EapH2. Residues of interest are drawn in ball-and-stick convention with proposed hydrogens bonds as dashed lines. Residues from NE (green) are labeled with italicized letters, while residues from EapH2 (teal) are labeled with normal typeface. The position of the NE catalytic serine is highlighted with an asterisk (i.e. *S201**). (E) Image of the CG/EapH2/NE ternary complex drawn as a molecular surface. NE and CG appear as solid surfaces, while EapH2 is presented as a semi-transparent mesh. Approximately 30% of the ~6,240 Å² of surface area available in EapH2 is buried upon formation of the ternary complex. (F) Representation of the CG/EapH2/NE complex colored according to the B-factors of the final model. Positions of low B-factors appear thinner and are colored blue, while those with high B-factors appear thicker and are colored orange/red. Proteins are shown in an identical orientation to panels A, B, and E.

The CG/EapH2 interface buries 1,003 Å² of surface area with a shape complementarity coefficient (s.c.) of 0.74 (25). These values are comparable to the 1027 Å² of buried surface area and the s.c. of 0.73 that we previously reported for CG/EapH1 (15). As with EapH1, the interactions formed by EapH2 with CG arise primarily through mainchain-derived groups derived from both proteins (Fig. 6.4C & Fig. 6.1B). An extensive network of hydrogen bonds is formed by the polypeptide backbone from EapH2-G56 to EapH2-N66, which comprise 2-3 of the inhibitor. There is also a hydrogen bond between the backbone nitrogen of EapH2-N61 and the sidechain of the catalytic serine, CG-S196. Interestingly, the sidechains of EapH2-N61 and the adjacent position, EapH2-Q62, form additional hydrogen bonds with groups derived from CG. Presumably these interactions lead to increased rigidity at the CG/EapH2 interface. The most prominent sidechain-derived hydrogen bonds are made by EapH2-R90, however, which interacts

with the backbone carbonyls of both CG-Q97 and CG-R98. The significance of these contacts is underscored by our kinetic studies on the EapH2-R90M, which displays an ~17.8-fold loss of function when compared to the wild-type inhibitor (Table 6.2).

The NE/EapH2 interface buries 882 Å² of surface area with an s.c. of 0.75. Again, these values are comparable to the 868 Å² of buried surface area and s.c. of 0.77 for NE/EapH1 (3). Consistent with our earlier NMR titration studies (c.f. Fig. 6.3D), the interactions formed by EapH2 with NE involve groups from both its polypeptide backbone and sidechains and are found in three distinct regions of the inhibitor (Fig. 6.4D & Fig. 6.1B). These are EapH2-Q72 to EapH2-L76 and EapH2-W109 to EapH2-K115, both of which form a single sidechain-mediated hydrogen bond with NE (i.e. EapH2-Q72 and EapH2-S113, respectively), along with EapH2-K121 to EapH2-S133, which comprises 6-8 of the inhibitor and accounts for seven different hydrogen bonds and/or salt bridges with NE. This includes a hydrogen bond with the catalytic serine, NE-S201, by the backbone carbonyl of EapH2-A126; however, this hydrogen bond involves the mainchain nitrogen instead of the NE-S201 sidechain. Other noteworthy interactions are formed by the sidechains of EapH2-N127, which hydrogen bonds to the backbone carbonyl of NE-H69, and of EapH2-D122, which forms a salt bridge with the sidechain of NE-R219. The importance of these contacts has already been established through our earlier studies on the EapH2-N127A and EapH2-D122A mutants, which display an ~188-fold and ~12.3-fold loss of function when compared to wild-type (14).

An additional contact also seems to be made by the sidechain of EapH2-D130, which appears to form a salt bridge with the sidechain of NE-R49. This residue was not implicated in our earlier model of the NE/EapH2 complex. To test the importance of this newly identified interaction, we prepared an EapH2-D130A mutant and assessed its impact on NE inhibition by

EapH2. This mutant displays activity almost identical to wild-type EapH2 ($K_i=2.5$ nM) (Table 6.2). To further explore any potential role, we constructed an EapH2-D122A/D130A double mutant, since these acidic residues lie at opposing ends of the loop that comprises a bulk of the EapH2 interface with NE (Fig. 6.4D). This mutant has features that mirror those of the EapH2-D122A mutant characterized previously (14), as it displays an ~ 10.5 loss of function when compared to wild-type (Table 6.2). Therefore, the sidechain contact of EapH2-D130 does not seem to be a major factor in NE inhibition by EapH2.

EapH2, but not EapH1, is Capable of Inhibiting CG and NE Simultaneously

The CG/EapH2/NE structure shows that a single EapH2 polypeptide has two unique binding modes that can occlude the active sites of CG and NE concurrently (Fig. 6.4). However, to establish whether ternary complexes like CG/EapH2/NE could simultaneously inhibit CG and NE activity in solution, we compared the progress curves and extent of inhibition of reactions containing both NSP enzymes, their substrates, and EapH2 with reactions consisting of only one NSP, both protease substrates, and EapH2 (Fig. 6.5). We performed an analogous series of reactions with EapH1 as a control, since EapH1 relies on a single binding mode to block CG and NE that cannot be occupied by both NSPs concurrently (Fig. S1A-B). To facilitate interpretation of the data, we employed a colorimetric substrate for CG (i.e. SA₂PF-pNA) and a fluorescent substrate for NE (i.e. MSA₂PV-AMC).

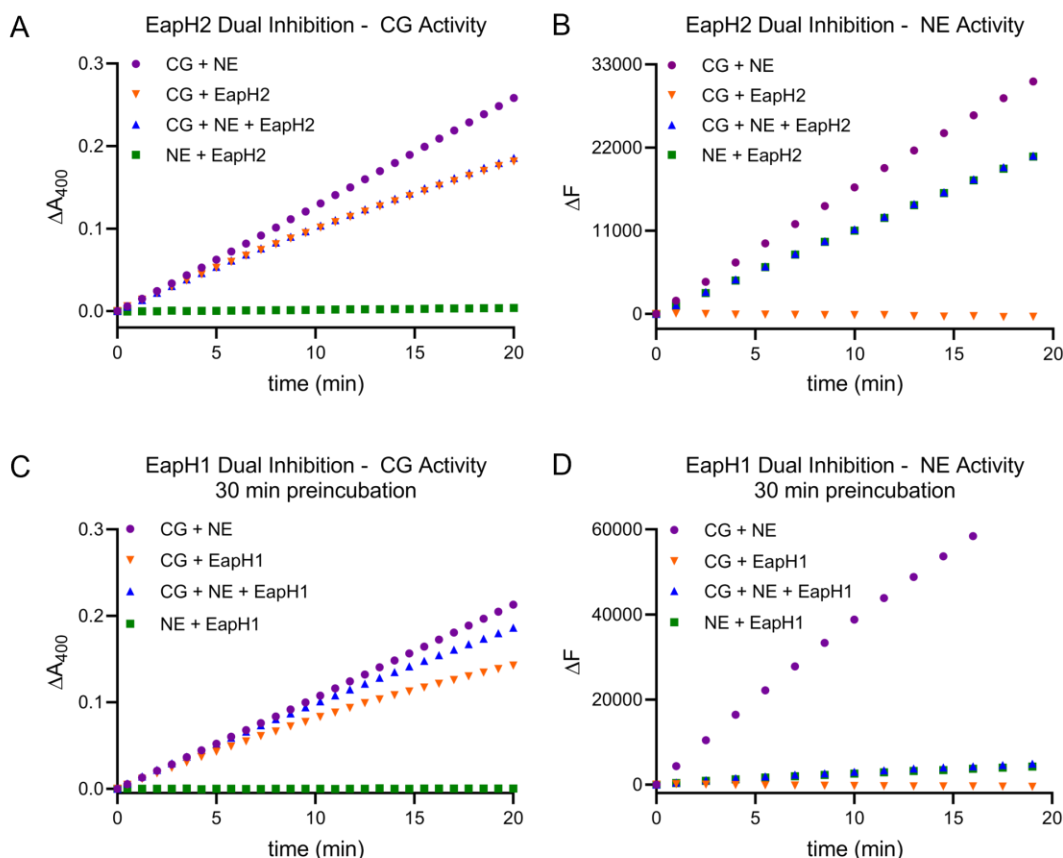


Figure 6.5 Demonstration of simultaneous inhibition of CG and NE by EapH2 but not EapH1.

The potential for simultaneous inhibition of CG and NE by EAP proteins was examined using pairwise reaction sequences where CG activity was followed by absorbance and NE activity was followed by fluorescence. Representative data from one of two experimental series are shown. (A) Inhibition of CG and NE by EapH2 followed by SA₂PF-pNA turnover by CG. (●) Reaction consists of 7.5 nM CG, 7.6 nM NE, 0 nM EapH2. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 8.1 nM EapH2. (▲) Reaction consists of 7.5 nM CG, 7.6 nM NE, 8.1 nM EapH2. (■) Reaction consists of 0 nM CG, 7.6 nM NE, 8.1 nM EapH2. (B) Inhibition of CG and NE by EapH2 followed by MSA₂PV-AMC turnover by NE. (●) Reaction consists of 7.5 nM CG, 7.6 nM NE, 0 nM EapH2. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 8.1 nM EapH2. (▲) Reaction consists of 7.5 nM CG, 7.6 nM NE, 8.1 nM EapH2. (■) Reaction consists of 0 nM CG, 7.6 nM NE, 8.1 nM EapH2. (C) Inhibition of CG and NE by EapH1 followed by SA₂PF-pNA turnover by CG. (●) Reaction consists of 7.5 nM CG, 20.2 nM NE, 0 nM EapH1. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 19.5 nM EapH1. (▲) Reaction consists of 7.5 nM CG, 20.2 nM NE, 19.5 nM EapH1. (■) Reaction consists of 0 nM CG, 20.2 nM NE, 19.5 nM EapH1. (D) Inhibition of CG and NE by EapH1 followed by MSA₂PV-AMC turnover by NE. (●) Reaction consists of 7.5 nM CG, 7.3 nM NE, 0 nM EapH1. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 19.5 nM EapH1. (▲) Reaction consists of 7.5 nM CG, 20.2 nM NE, 19.5 nM EapH1. (■) Reaction consists of 0 nM CG, 20.2 nM NE, 19.5 nM EapH1. Note that the substrate concentrations in all reactions were 1.175 mM SA₂PF-pNA and 170 μM MSA₂PV-AMC.

We found that the extent of CG inhibition upon concurrent exposure of the enzyme to its substrate and EapH2 is unchanged by the presence of NE (Fig. 6.5A). Similarly, we found that NE inhibition is unchanged upon concurrent exposure of the enzyme to its substrate and EapH2 by the presence of CG (Fig. 6.5B). The simplest interpretation of these results is that EapH2 binding to CG is not affected by NE and, conversely, that EapH2 binding to NE is not affected by CG. These observations are consistent with the ability of EapH2 to bind both NSPs simultaneously, as judged by the structure of the CG/EapH2/NE ternary complex (Fig. 6.4), and also with the NMR titration studies which show that the two NSP-binding sites in EapH2 function independently of one another (Fig. 6.3). By contrast, we found that CG activity is restored upon inclusion of NE in the reaction when we performed the control experiment with EapH1 (Fig. 6.5C). Although we did not observe recovery of NE activity in the corresponding experiment (Fig. 6.5D), the apparent affinity of EapH1 for NE is ~2,000-fold higher than for CG (Table 6.2; (13)). This strongly suggests that EapH1 will remain associated with NE under these conditions. The simplest interpretation of these results is that EapH1 binding to CG is impacted by NE; although the converse cannot be inferred directly from these results, the loss of EapH1 potency toward CG in the presence of NE demonstrates that EapH1 can only bind one NSP at a time. Together, these results provide evidence that EapH2 can simultaneously bind and inhibit CG and NE in solution, but that EapH1 cannot. Combined with the structural studies presented in the sections above, we propose that EapH2 defines a new mechanism for bifunctional inhibition of NSPs.

Discussion

A combination of features has been attributed to EAP proteins that make them unique among biological protease inhibitors. EAP proteins do not share apparent sequence or structural

relationships to other well-characterized protease inhibitor families (3). Instead, they share a β -grasp fold similar to other secreted superantigen toxins and immune evasion proteins of *S. aureus* (7, 8). This suggests that EAP proteins arose through convergent evolutionary processes, presumably in response to selective pressure on *S. aureus* necessitating inhibition of host NSPs (2). Consistent with this explanation, EAP proteins show high selectivity for the serine proteases found in neutrophil granules, as opposed to other chymotrypsins that function elsewhere in the immune system or in the bloodstream (3). This stands in contrast to more widely distributed protease inhibitors, most notably SERPINs, which target multiple serine proteases with broad effect. Furthermore, despite their high affinities, EAP proteins inhibit NSPs by forming reversible, non-covalent complexes (3, 13-15, 24). This culminates in an unusual, time-dependent inhibitory character (13-15, 24), although the physical basis for and biological significance of this property are not known. Therefore, we have undertaken a series of structure/function studies to better understand the molecular features that underlie NSP inhibition by EAP proteins.

Our initial work on EapH2 suggested that this EAP protein might use an alternative binding mode to inhibit NE as compared to that used by EapH1 (14, 16). Unfortunately, our inability to crystallize EapH2 bound to NE precluded us from defining the structural features of this proposed alternative binding mode. Similarly, the structural basis for EapH2 inhibition of CG remained unknown even though this activity was described in the initial report that identified EAP proteins as NSP inhibitors (3). To address these questions through an alternative approach, we used solution NMR titration studies to monitor changes in the chemical shifts of residues in EapH1 and EapH2 in response to increasing concentrations of CG and NE (Fig. 6.3 & Fig. S2-4). Our results showed that corresponding residues in EapH1 and EapH2 experience chemical shift perturbations in the presence of CG (Fig. 6.3A-B), but that distinct regions of EapH1 and EapH2 are affected by the

presence of NE (Fig. 6.3C-D). The apparent NSP-binding sites identified by our NMR titration analysis are consistent with available crystal structures of NE/EapH1 and CG/EapH1 (Fig. 6.1B & Fig. S1A-B) (3, 15), site directed mutagenesis studies on EapH1 and EapH2 described in previous publications (Fig. 6.1B) (13, 14), and the differences in CG inhibition between EapH2 and EapH2-R90M that we describe here (Fig. 6.2 & Table 6.2). Further evaluation of these data suggested to us that EapH2 might contain independent binding sites for CG and NE that could be occupied simultaneously, which we demonstrated by solving the crystal structure of a CG/EapH2/NE ternary complex (Fig. 6.4 & Fig. S5). Thereafter, we showed that CG inhibition by EapH2 is not altered by the presence of NE and vice versa (Fig. 6.5A-B), but that this is not the case for EapH1 (Fig. 6.5C-D). Thus, the results we describe here provide both structural and biochemical evidence for simultaneous blockade of two NSPs by a single functional domain, EapH2.

CG and NE share a common chymotrypsin fold, yet are only 34% identical at the sequence level (15). Contrary to what we described for EapH1 (3, 15), we found that these two proteases are recognized by distinct binding sites on EapH2 (Fig. 6.1B & Fig. 6.4A-B). Furthermore, the nature of the interactions formed by EapH2 with CG and NE also differ in some respects (Fig. 6.4C-D). First, although EapH2 engages in numerous hydrogen bonds with mainchain derived groups from both CG and NE, the total of 17 hydrogen bonds at the CG binding site is somewhat higher than the 13 hydrogen bonds at the NE binding site, as determined by EBI-PISA analysis (26). This may be a contributing factor to the larger buried surface area at the CG/EapH2 interface ($1,003 \text{ \AA}^2$) as compared to NE/EapH2 ($882 \text{ \AA}^2$). Second, while EapH2 interacts directly with groups derived from the catalytic serine of both proteases, the details of these interactions differ between binding sites. At the CG-binding site, EapH2-N61 uses its mainchain nitrogen to hydrogen bond to the sidechain of CG-S196, whereas at the NE-binding site, EapH2-A126 uses its mainchain carbonyl

to hydrogen bond to the mainchain nitrogen of NE-S201. Finally, whereas EapH2 makes use of sidechain-derived interactions to contact both CG and NE, the interactions at the NE interface involve more extensive contacts with sidechains derived from the protease. Whereas there is only a single sidechain-sidechain hydrogen bond between EapH2 and CG (i.e. EapH2-N61 to CG-S42), there are four sidechains in EapH2 that combine to form seven different hydrogen bonds and salt bridges with sidechains of NE, as judged by EBI-PISA analysis (26). One of these positions, EapH2-D122, results in ~12.3-fold loss in NE inhibitory activity upon mutation to alanine (14).

There are other noteworthy structural features of the CG/EapH2/NE ternary complex. One feature concerns the total buried surface area for EapH2 in its free versus the fully occupied form (Fig. 6.4E). In its unbound state, EapH2 presents ~6,240 Å² of surface area. Accounting for the CG/EapH2 (1,003 Å²) and NE/EapH2 (882 Å²) interfaces described above, the ternary complex buries a total of ~1,885 Å². This corresponds to ~30% of the total surface area of EapH2, which seems especially high since EapH2 is a globularly shaped protein. Considering this large percentage of buried surface area, along with the abundance of charged sidechains in EapH2, we suspect that NSP binding at both sites would likely involve significant loss of ordered solvent from EapH2. Absent other factors, it seems that this could provide an entropic driving force to formation of the CG/EapH2/NE ternary complex. In the future, it might be informative to investigate this issue directly using a suitable method, such as isothermal titration calorimetry. Unfortunately, calorimetry is a material intensive technique, and the lack of plentiful, recombinant sources for these NSPs is a practical barrier to performing such studies at the present time.

A second interesting feature relates to the distribution of B-factors in the final CG/EapH2/NE model (Fig. 6.4F). Whereas CG itself and EapH2 positions closer to the CG-binding site exhibit relatively low B-factors, those of NE as well as the EapH2 positions near the

NE-binding site show much higher overall B-factors. To determine if this is a *bona fide* feature of the CG/EapH2/NE complex rather than something unique to the crystals grown at pH 5.5, we solved two additional CG/EapH2/NE structures from crystals grown at pH 7.5 and pH 8.5 (Fig. S5A-D & Table 6.3). Although the final models for each CG/EapH2/NE complex are basically indistinguishable from one another (Fig. S5E), the trend toward significantly higher B-factors in NE and its binding site in EapH2 is retained independent of crystallization conditions (Fig. S5F). Unfortunately, the significance of these observations is not immediately evident. Furthermore, the lack of CG/EapH2 and NE/EapH2 complex structures limits our ability to interpret whether this is due to EapH2 binding NE or forming the CG/EapH2/NE ternary complex specifically. Examining the cocrystal structures of other EAP proteins bound to NE or CG does provide some clues, though. NE, when inhibited by EapH1, or either the first (Eap1) or second (Eap2) domains of the *S. aureus* Extracellular Adherence Protein, does not show unusually high B-factors (3, 24), nor does CG when inhibited by the same three EAPs (15, 24). Since this portfolio of structures represents both NSP-binding modes identified for EAP proteins (3, 15, 24), it does not seem that the elevated B-factors arise simply from NE binding to the site utilized by EapH2, Eap1, and Eap2. By contrast, EapH1, Eap1, and Eap2 all show elevated B-factors in regions distal to the occupied NSP-binding site, regardless of the protease being inhibited (3, 15, 24). This suggests that EAP domain residues away from an occupied NSP-binding site might undergo structural and/or dynamic changes as a consequence of binding, as we previously proposed (24). Therefore, it is interesting to speculate that the elevated B-factors of NE in the CG/EapH2/NE complex arise from propagation of these changes across this unusual ternary complex. While obtaining experimental evidence for this sort of phenomenon would likely be difficult, we think that comparative studies on binary and ternary

NSP/EAP domain complexes using hydrogen/deuterium exchange mass spectrometry could be useful in such efforts.

Much of our insight into EAP proteins as NSP inhibitors has arisen through studies on EapH1 (2, 3, 13-16). Nevertheless, information presented here and elsewhere suggests that it may now be appropriate to reconsider using EapH1 as a paradigm (24). EapH1 has a single NSP-binding mode that displays nanomolar-level inhibition of CG ($K_I = 43.7$ nM), but which inhibits NE ~2,000-fold more potently ($K_I = 21$ pM) (13,15). By contrast, EapH2 has separate CG- and NE-binding modes that differ by only ~10-fold in their inhibitory potency (c.f. $K_{I,CG} = 25.4$ nM vs $K_{I,NE} = 2.1$ nM) (Table 6.2). Our structural observations also show that these two NSP-binding sites within EapH2 function independently of one another (Figs. 6.3 & 6.4). As mentioned earlier, we recently reported the CG- and NE-bound structures of two other EAP proteins, known as Eap1 and Eap2, along with characterization of their abilities to inhibit CG and NE (24). Both Eap1 and Eap2 utilize independent CG- and NE-binding sites that are analogous to those of EapH2, and also compatible with formation of EapH2-like ternary complexes (Fig. S1F) (24). The inhibitory potencies of Eap1 (c.f. $K_{I,CG} = 8.5$ nM vs $K_{I,NE} = 240$ pM) and Eap2 (c.f. $K_{I,CG} = 6.9$ nM vs $K_{I,NE} = 500$ pM) likewise match those of EapH2 more closely when compared to EapH1 (15, 24). Considering that Eap1 and Eap2 exhibit EapH2-like structure/function relationships, we propose that EapH2 is a more suitable paradigm for understanding NSP inhibition by EAP proteins than EapH1.

To our knowledge, simultaneous inhibition of two serine proteases by a single functional domain is unprecedented. Although there are several potential implications of this finding, we believe it is particularly interesting to consider that neither the N- nor C-terminus of EapH2, nor those of Eap1 or Eap2, appear to be impacted by formation of ternary complexes with NSPs. Both

Eap1 and Eap2 are domains found within the larger, multidomain Extracellular Adherence Protein of *S. aureus* (8, 24, 27). We have shown that the four-domain isoform of this molecule expressed by *S. aureus* strain Mu50 adopts an elongated, but structured conformation in solution that is facilitated by the N- and C-termini lying at opposite poles of the individual EAP domains (27). Since each of these domains contains two potent and independent NSP-inhibitory activities (3, 24), we suspect that the secreted Extracellular Adherence Protein of *S. aureus* may function as an unusual polyvalent protease inhibitor capable of neutralizing one equivalent of NE and one equivalent of CG for each EAP repeat in its sequence. Future studies will be designed to explore this possibility directly.

What might be the specific roles of Eap, EapH1, and EapH2 in biological settings, given their unique structure/function and NSP-inhibitory properties? Production of certain immune evasion proteins, including Eap and EapH1, is upregulated following exposure of *S. aureus* to neutrophil azurophilic granule components (28). In human neutrophil azurophilic granules, NE and CG are the two most abundant NSPs with NE present in a slight excess (29). Although neutrophils can release their granular contents into the extracellular environment, engulfing bacteria like *S. aureus* and enclosing them within the phagosome provides for far more effective killing (30): once the granular contents are released into the phagosome, the stage is set for functional synergy between oxidative and non-oxidative anti-microbial systems (31). Together, these observations suggest that EAPs most likely act within the neutrophil phagosome. In that regard, it seems that EapH1 is as a preferential inhibitor of NE, owing to its having only a single NSP-binding site that exhibits orders of magnitude greater potency toward NE. Whereas the properties of EapH2 suggest that it may function as either an inhibitor of NE or CG alone, the high concentrations of NSPs believed to exist within the neutrophil phagosome (i.e. >1 mM (29, 32))

would be conducive to further forming the ternary NSP-inhibitory complex described in this report. This same argument would apply to the multidomain Eap since it appears to follow similar structure/function relationships and have comparable biochemical parameters to EapH2, at least as far as NSP inhibition is concerned (11, 12). Completing this picture will require a deeper understanding of how EAPs inhibit the third canonical NSP, known as Proteinase-3, and better defining the levels of EAPs produced by *S. aureus* prior to and following phagocytosis. Recognizing these limitations, we propose that it is this potent and concerted inhibition of multiple NSPs, rather than inhibition of any single NSP alone, which underlies earlier observations that established a role for all three EAPs in promoting staphylococcal infection (3).

Experimental Procedures

Protein samples

Human neutrophil CG was purchased from Innovative Research. Human NE was purchased from Elastin Products Company. All derivatives of the prokaryotic expression vector pT7HMT (33) that encode either wild-type or mutant forms of EapH1 and EapH2 were obtained from GenScript USA, Inc. All forms of EapH1 and EapH2 were expressed using *E. coli* BL21(DE3) cells and purified as described in earlier publications (13-15). Isotopically-enriched samples for use in NMR spectroscopy studies were obtained using the general procedures outlined above but with the modifications given by Woehl et al. (34). The concentrations of all EapH1 and EapH2 proteins were determined by UV absorbance using extinction coefficients calculated from the expected protein composition by ExPASy ProtParam (35, 36).

Preparation of CG, NE, and substrates

Human CG was stored at 4 °C in a buffer of 50 mM sodium acetate/800 mM NaCl (pH: 5.5). The concentration of CG was determined from its expected protein composition. The enzyme was diluted into 20 mM HEPES/140 mM NaCl/0.05% (v/v) Tween-20 (pH: 7.4) immediately prior to use in activity assays. The chromogenic substrate Succinyl-Ala-Ala-Pro-Phe-p-nitroanalide (SA₂PF-pNA) was purchased from Cayman Chemical Company and dissolved in 100% (v/v) DMSO. Owing to the hygroscopic nature of DMSO (37), all aliquots of solvent and prepared substrate were stored at 4 °C in a container containing desiccant when not in use.

Lyophilized human NE (1 mg) was dissolved in 200 µL of 20 mM sodium acetate/50% (v/v) glycerol (pH: 5.0). The concentration of NE was determined from its expected protein composition. The enzyme was stored in 10 µL aliquots at -20 °C until use. The chromogenic substrate methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanalide (MSA₂PV-pNA) was purchased from Sigma-Aldrich and dissolved in 100% (v/v) DMSO. End-point assays ($\epsilon_{400}=12.3 \text{ mM}^{-1}\text{cm}^{-1}$) were used to quantify substrate concentrations following dilution with 50 mM HEPES/140 mM NaCl/0.05% (v/v) Tween-20 (pH: 7.4). The fluorescent substrate, methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin (MSA₂PV-AMC), was likewise purchased from Cayman Chemical Company. It was dissolved in 100% (v/v) DMSO to a final concentration of 15.9 mM.

Reversible behavior of CG inhibition by EapH2

The reversibility of CG inhibition by EapH2 was investigated as previously reported (15). Briefly, wild-type EapH2 (13.45 µM) was pre-incubated with 900 nM CG for 30 min. The complex was then diluted into an assay mixture (1.14 mM SA₂PF-pNA in 50 mM HEPES/140 mM NaCl/0.025% (v/v) Tween-20 (pH: 7.4)) with 26.18% (v/v) DMSO) to yield final concentrations

of 154 nM EapH2 and 10.3 nM CG. Finally, the absorbance was followed at 400 nm for 150 min. As a control, a separate series of reactions were followed at the same concentrations but did not employ preincubation of CG with EapH2.

Steady-state kinetic analysis of CG and NE

Reaction progress curves were collected for 30 min using a Molecular Devices Versa_{Max} tunable microplate reader. A total of 30 progress curves were collected for each CG inhibitor: two CG concentrations (7 and 12 nM), three substrate concentrations ($\sim 0.4K_m$, $0.7K_m$, and $1.1K_m$) per CG concentration, and five inhibitor concentrations per substrate concentration. The data were fit to a rapid-equilibrium competitive inhibition mechanism using KinTek Global Kinetic Explorer (38, 39), as outlined in earlier publications (Fig. S6) (13, 14, 24). The search space for progress curve fitting was decreased by linking the substrate off-rate (k_2) to the on-rate by the estimated Michaelis constant (K_m). This value has been previously determined by steady-state kinetic analysis using a microplate-based assay (13, 15). The apparent inhibition constant (K_I) was calculated from the on- and off-rate estimates determined from the fitting procedure given above. The values reported are the average of at least three different sets of data, with the standard deviation in each parameter representing the reported errors.

An analogous procedure was used to monitor inhibition of NE by EapH2 as outlined in previous publications (13, 14, 24). In this case, a total of 45 progress were collected for each NE inhibitor: three NE concentrations (4, 7.9, and 10.6 nM), three substrate concentrations ($\sim K_m$, $2K_m$, and $3K_m$) per NE concentration, and five inhibitor concentrations per substrate concentration. The data were fit to the same mechanism used for CG using KinTek Global Kinetic Explorer (Fig. S6) (38, 39).

Simultaneous inhibition of CG and NE by EapH1 and EapH2

To probe the ability of EAP proteins to inhibit both NSPs simultaneously, parallel reaction pairs were prepared consisting of both proteases with their respective substrates with or without the EAP inhibitor. The CG reaction was followed spectrophotometrically (400 nm) while the NE reaction was followed fluorometrically (λ_{ex} : 355 nm, λ_{em} : 455 nm). The buffer consisted of 50 mM HEPES/140 mM NaCl/0.05% (v/v) tween-20/5% (v/v) DMSO (pH: 7.4). To ensure rigor and reproducibility, all experimental series were repeated twice.

To investigate simultaneous inhibition by EapH2, four separate pairs of reactions were prepared. Reaction pair one consisted of 15 nM CG with 340 μ M MSA₂PV-AMC in the top well and 15.2 nM NE with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair two consisted of 15 nM CG with 340 μ M MSA₂PV-AMC in the top well and 16.2 nM EapH2 with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair three consisted of 15 nM CG with 340 μ M MSA₂PV-AMC in the top well and 15.2 nM NE with 2.35 mM SA₂PF-pNA and 16.2 nM EapH2 in the lower well. Reaction pair four consisted of 340 μ M MSA₂PV-AMC in the top well and 15.2 nM NE with 2.35 mM SA₂PF-pNA and 16.2 nM EapH2 in the lower well. The reactions were initiated by mixing 170 μ L of the top well into 170 μ L of the lower well. The final concentrations of the reaction components were 7.5 nM CG, 7.6 nM NE, 8.1 nM EapH2, 170 μ M MSA₂PV-AMC and 1.175 mM SA₂PF-pNA.

To investigate simultaneous inhibition by EapH1, four separate reaction pairs were also prepared. Reaction pair one consisted of 15 nM CG incubated with 340 μ M MSA₂PV-AMC in the top well with 40.4 nM NE incubated with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair two consisted of 15 nM CG incubated with 340 μ M MSA₂PV-AMC in the top well with 39 nM EapH1 incubated with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair three consisted of

15 nM CG incubated with 340 μ M MSA₂PV-AMC in the top well with 40.4 nM NE incubated with 2.35 mM SA₂PF-pNA and 39 nM EapH1 in the lower well. Reaction pair four consisted of 340 μ M MSA₂PV-AMC in the top well with 40.4 nM NE incubated with 2.35 mM SA₂PF-pNA and 39 nM EapH1 in the lower well. The reaction components were allowed to incubate for 30 min prior to mixing. The final concentrations of the reaction components were 7.5 nM CG, 20.2 nM NE, 19.5 nM EapH1, 170 μ M MSA₂PV-AMC and 1.175 mM SA₂PF-pNA.

NMR spectroscopy

All proteins were reconstituted in NMR buffer (50 mM sodium phosphate/50 mM NaCl/ 5 % (v/v) D₂O (pH: 6.5)) and quantified prior to experimentation. Solution NMR spectra were collected at 25 °C using either a 700 MHz Agilent DD2 NMR system or an 800 MHz Bruker Avance III NMR spectrometer (for the purposes of resonance assignment), or a 600 MHz Bruker Avance Neo NMR instrument (for the purposes of titration studies). All instruments were equipped with a 5 mm HCN triple resonance cryogenically cooled probe. All triple resonance NMR experiments were acquired using non-uniform sampling. Uniformly sampled data were obtained using reconstruction methods such as hmsIST, SMILE, NMRPipe IST, and RIST as implemented in the NMRPipe software (40).

For solution structure determination of EapH1 and EapH2, samples were reconstituted as described above and 200 μ L were transferred to a Wilmad 3 mm NMR tube. Data were collected using the instruments described above using Bruker TOPSPIN. The NMR spectra were processed using NMRPipe and analyzed with CARA (40). The mainchain and a portion of the sidechain chemical shifts were assigned using 2D ¹H-¹⁵N HSQC and standard set of triple resonance experiments including 3D HNCO, HNCA, HNCACB, HN(CA)CO, and HN(CO)CA. The

remaining sidechains were assigned using TOCSY and NOE-based experiments such as Dipsi HSQC, (H)C(CCO)NH, H(CCCO)NH, and ^{15}N -NOESY. NOESY experiments for EapH1 were acquired with both 80 and 100 ms mixing times, while for EapH2 only a 100 ms mixing time was used. Peak picking and resonance assignments were performed using CARRA NMR software and the assignments were used to predict dihedral angles and secondary structure using TALOS-N (41). The software Cyana v2.1 was used for both automated NOE assignments and structure calculations (42). H-bonding constraints were introduced during the final round of structure calculations and were experimentally derived from 2D ^1H - ^{15}N HSQC spectra collected for a period of 29 hours on samples dissolved in 100% (v/v) D_2O . The 20 lowest energy structures of the 200 total structures calculated were selected for preparation of the final structural ensemble. The programs Cyana 2.1, PyMOL (43), PROCHECK_NMR (44), and the Protein Structure Validation Software Suite (PSVS) were used to assess the quality of the structures. Chemical shifts, structural restraints (distance, dihedral, and H-bonding restraints), and atomic coordinates have been deposited in the BMRB and PDB using entry numbers 50304 and 8GDH for EapH1 and entry numbers 27540 and 8GDG for EapH2. Further information on NMR data collection and the properties for each structural ensemble can also be found in Table 6.1.

For chemical shift perturbation (CSP) experiments, NE and CG proteins were first lyophilized and reconstituted into 10 mM acetate buffer at pH 4.5 and 5.5, respectively. Uniformly $^{15}\text{N}/^{13}\text{C}$ double-labeled EapH1 and EapH2 were diluted in NMR buffer to 100 μM final concentration, while NSPs were added immediately before experimentation. Each experimental sample consisted of ~ 200 μL total volume. Titration studies were performed at 0, 25, 50, and 75 μM concentrations of NSPs; since these values are greater than 1,000-fold higher than the K_D/K_I of the interactions under investigation, these concentrations equate to 0, and ~ 25 , ~ 50 , and $\sim 75\%$

occupancy for the 100 μ M EapH1 and EapH2 in each sample. Freshly prepared samples were used at each titration point to avoid protein aggregation. 2D ^1H - ^{15}N HSQC spectra of uniformly $^{15}\text{N}/^{13}\text{C}$ labeled EapH1 and EapH2 were monitored to probe the residues of EAP proteins involved in the interaction with each NSP, though in certain instances 3D HNCO spectra were also used to confirm HSQC peak shifts. Signal intensities for original peak positions were plotted to obtain initial conjectures of peak shifts. Peak shifts were subsequently characterized by superimposing the HSQC spectrum at a given NSP concentration with that of an HSQC containing no NSP. The combined chemical shift changes were calculated using the following formula (23), where ΔH is the ^1H shift and ΔN is ^{15}N shift:

$$\text{Chemical Shift Perturbation } (\Delta\delta) = \sqrt{(\Delta H)^2 + \left(\frac{\Delta N}{6.51}\right)^2}$$

The HSQC data demonstrated that the peaks shifted from the free to the bound form in the slow exchange regime without any intermediate exchange. This was consistent with previous SPR studies and functional analyses presented here and elsewhere, which show high affinity (i.e. K_D or K_I) of EAP proteins toward both NE and CG (Table 6.2) (13-16, 24).

Protein crystallography

Samples of CG/EapH2/NE for crystallization were prepared by mixing an equimolar ratio of the individual molecules. All samples were buffer exchanged into a solution of 10 mM HEPES/50 mM NaCl (pH: 7.4) and concentrated to 5-10 mg/ml total protein, as judged by the anticipated extinction coefficient of the ternary complex. Initial crystallization conditions were identified by vapor diffusion of sitting drops prepared using a Crystal Gryphon liquid handling system (Art Robbins Instruments, LLC) along with the Index HT and PEGRx HT screening matrices (Hampton Research Corp.). Screening droplets consisted of 0.4 μ L total volume, prepared

as both 3:1 and 2:2 ratios of protein sample to precipitant solution, that were equilibrated aside 100 μ L of precipitant solution. When necessary, individual crystallization conditions were optimized by vapor diffusion of hanging drops that were prepared to systematically vary the buffer pH and precipitant concentration. All crystallization screening and optimization studies were conducted at 20 °C. Information on the final crystallization conditions and the composition of models is included in the paragraphs below.

X-ray diffraction data were collected on cryopreserved samples using beamline 22-ID of the Advanced Photon Source at Argonne National Laboratory. All X-ray diffraction data were indexed, integrated, and scaled using the HKL-2000 software package (45). Each structure was solved by molecular replacement using PHASER (46) as implemented in the PHENIX suite (47). PDB entries 1AU8, 1HNE (48), and 1YN5 (8) served as search models for CG, NE, and EapH2, respectively. Initial structure solutions were improved through a combination of automated rebuilding using PHENIX.AUTOBUILD (47) and manual rebuilding using COOT (49). All crystallographic models were refined by employing a combination of simulating annealing, reciprocal and real space positional, B-factor, and TLS refinement using PHENIX.REFINE (47). Each of the refined models and associated structure factors have been deposited in the PDB using the accession codes indicated in Table 6.3. Further information on X-ray diffraction data collection statistics and the properties for each structural model can also be found in Table 6.3.

Crystals of CG/EapH2/NE at pH 5.5 were obtained from a hanging drop that had been prepared by mixing 1 μ L of 5 mg/ml protein with 1 μ L of a precipitant solution consisting of 0.1 M Bis-Tris, 0.2 M sodium chloride, 26% (w/v) peg-3350 (pH: 5.5). The crystal was cryoprotected prior to harvest by briefly soaking in a buffer of precipitant solution supplemented with an additional 10% (w/v) peg-3350. X-ray diffraction data extending to 1.82 Å limiting resolution

were collected, indexed, and scaled in the space group $P2_1$ using HKL-2000. The structure was solved by molecular replacement as described above and contains a single copy of the CG/EapH2/NE complex in the asymmetric unit. The final model consists of complete copies of CG, EapH2, and NE without chain breaks, a partial model of the N-linked glycans associated with NE, and 163 ordered solvent molecules. The final model and refined structure factors have been deposited in PDB as entry 8G24.

Crystals of CG/EapH2/NE at pH 7.5 were obtained from a hanging drop that had been prepared by mixing 1 μ L of 5 mg/ml protein with 1 μ L of a precipitant solution consisting of 0.1 M HEPES, 0.2 M lithium sulfate, 26% (w/v) peg-3350 (pH: 7.5). The crystal was cryoprotected prior to harvest by briefly soaking in a buffer of precipitant solution supplemented with an additional 10% (w/v) peg-3350. X-ray diffraction data extending to 1.80 Å limiting resolution were collected, indexed, and scaled in the space group $P2_1$ using HKL-2000. The structure was solved by molecular replacement as described above; it contains three copies of the CG/EapH2/NE complex related to one another by translational non-crystallographic symmetry. The final model consists of three complete copies of CG, EapH2, and NE without chain breaks along with 633 ordered solvent molecules. Using the ternary complex defined by chains A, B, and C as a basis for comparison, the other complexes superimpose with RMSD values of 0.186 Å (chains D, E, and F) and 0.100 Å (chains G, H, and I). The final model and refined structure factors have been deposited in the PDB as entry 8G25.

Finally, crystals of CG/EapH2/NE at pH 8.5 were obtained from a sitting drop that had been prepared by mixing 0.3 μ L of 5 mg/ml protein with 0.1 μ L of a precipitant solution consisting of 0.1M TRIS, 0.2 M magnesium chloride, 25% (w/v) peg-3350 (pH: 8.5). The crystal was cryoprotected prior to harvest by overlaying the mother liquor with 2 μ L of a buffer consisting of

precipitant solution supplemented with 10% (v/v) glycerol. X-ray diffraction data extending to 1.85 Å limiting resolution were collected, indexed, and scaled in the space group $P2_1$ using HKL-2000. The structure was solved by molecular replacement as described above and contains a single copy of the CG/EapH2/NE complex in the asymmetric unit. The final model consists of complete copies of CG, EapH2, and NE without chain breaks, a partial model of the N-linked glycans associated with NE, and 219 ordered solvent molecules. The final model and refined structure factors have been deposited in the PDB as entry 8G26.

Analyses of protein structures

Representations of protein structures were generated from PDB files using PyMol (Shrodinger, LLC) (43). The R.M.S.D. of superposition for various proteins or protein-protein complexes were likewise obtained using the Align feature of PyMol. Calculations of total surface areas, buried surface areas at interfaces, and identification of likely hydrogen bonds and salt bridges were carried out using EBI-PISA (26). Surface complementarity coefficients for protein-protein interactions were obtained through the program Sc (25) as implemented in the CCP4 suite (50). Protein sequence alignments and assessments of sequence similarity/identity were generated by Clustal Omega (36) and rendered using EsPript (51).

Data availability

The atomic coordinates and structure factors for crystal structure determinations (PDB codes: 8G24, 8G25, and 8G26) and for solution structure determinations (PDB codes: 8GDG and 8GDH) have been deposited in the PDB (<http://wwpdb.org/>) and are publicly available. Chemical shifts and structural restraints (distance, dihedral, and H-bonding restraints) have been deposited

in the BMRB (<https://bmr.bio.org/>) and are also publicly available. All other experimental data are available upon request by contact the corresponding author B.V.G. (geisbrechtb@ksu.edu).

Supporting Information

This article contains supporting information.

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Author Contributions Statement

N.B.M.: NMR spectroscopy and protein crystallography

T.J.H.: enzyme assays and protein crystallography

O.P.: NMR spectroscopy

B.V.G.: Protein crystallography, project design & administration, and wrote the manuscript

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Conflict of Interest Statement

The authorship team declares no competing financial interests.

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Supplementary information

Simultaneous inhibition of two neutrophil serine proteases by the
S. aureus innate immune evasion protein EapH2

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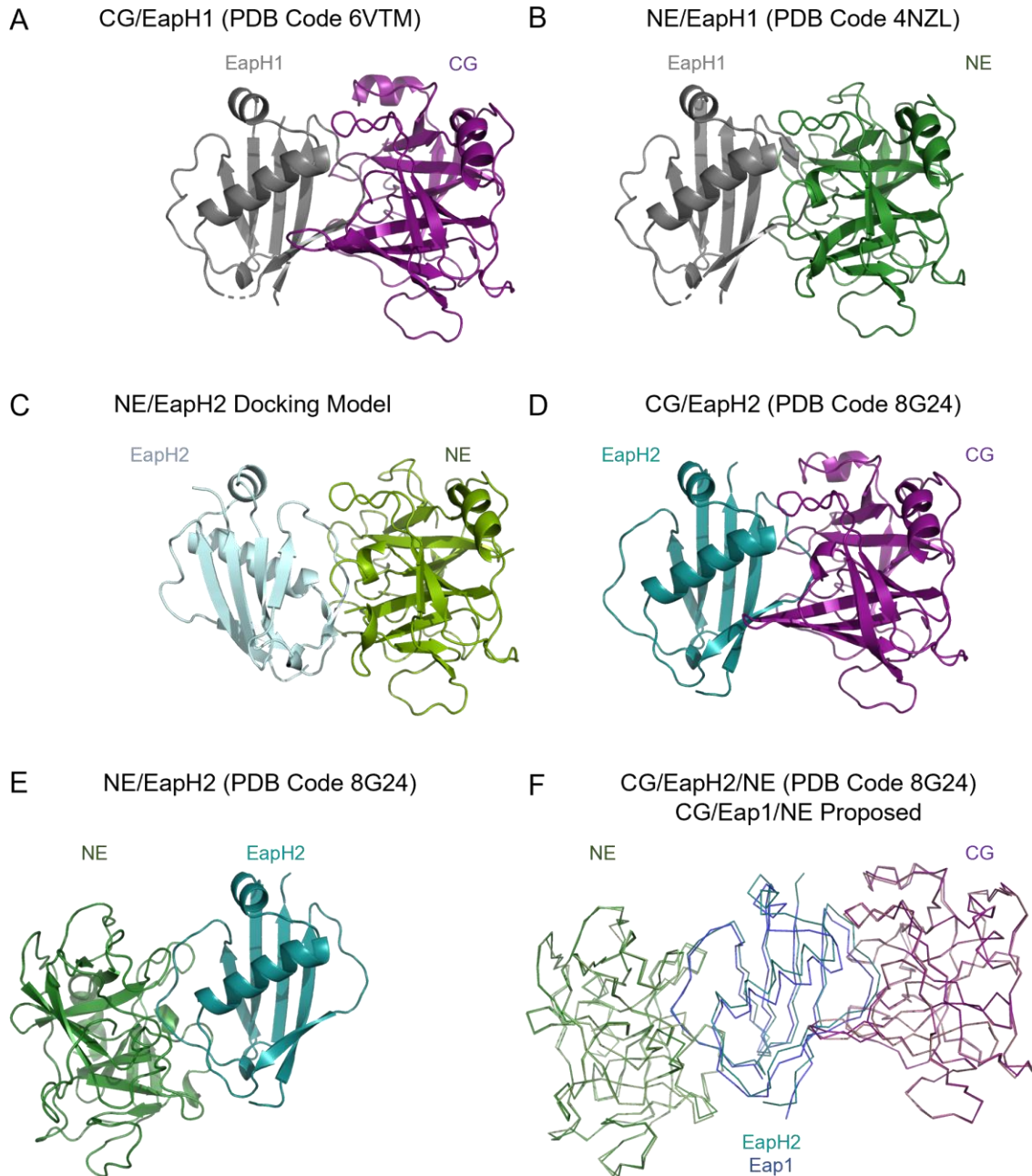
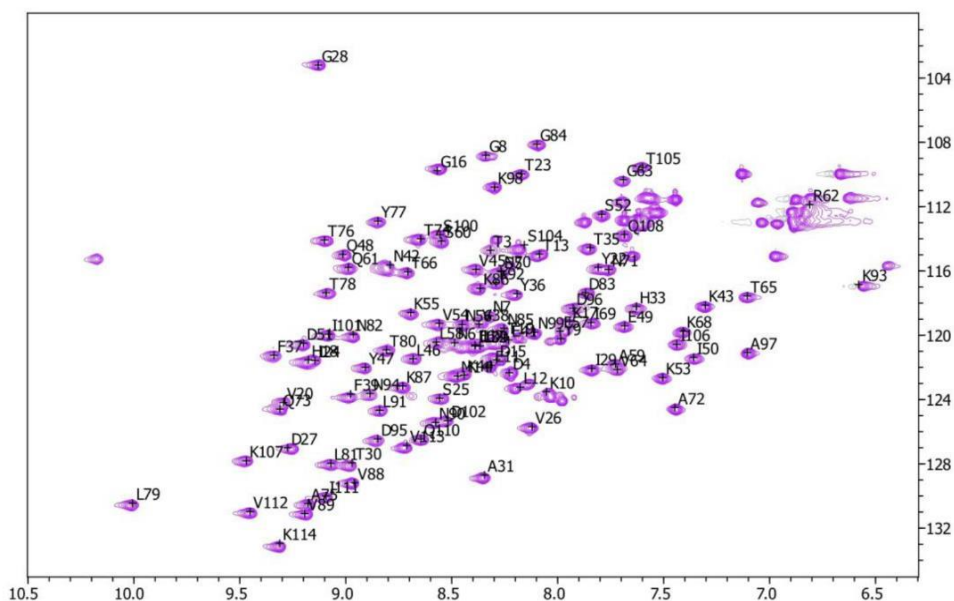


Figure S1. Comparison of binary complex models for CG and NE inhibited by EapH1 and EapH2. (A) Structure of CG (purple) inhibited by EapH1 (grey) as drawn from the PDB entry 6VTM. (B) Structure of NE (green) inhibited by EapH1 (grey) as drawn from the PDB entry 4NZL. (C) Computational model of the NE (light green) complex with EapH2 (light blue) described previously. Both NE and EapH2 are drawn with different colors than the other panels to signify this is the outcome of modeling, rather than empirical structure determination. Note the alternative orientation of the EAP domain relative to the protease in this structure. (D) Structure of CG (purple) inhibited by EapH2 (teal) as drawn from the PDB entry 8G24. (E) Structure of NE (green)

inhibited by EapH2 (teal) as drawn from the PDB entry 8G24. (F) Structure of CG (light purple) inhibited by Eap1 (blue) as drawn from the PDB entry 8D4S, along with the structure of NE (light green) inhibited by Eap1 (blue) as drawn from the PDB entry 8D4Q, superimposed upon the structure of the CG (purple), EapH2 (teal), NE (green) complex as drawn from PDB entry 8G24. For simplicity, proteins are represented as C α wire and only a single copy of Eap1 is shown. Note that the orientations of the EAP domain proteins are held constant in all panels A, B, D, E, and F, while the orientations of the NE proteins are held constant in panels B and C.

A ^1H - ^{15}N HSQC Spectrum of EapH1



B ^1H - ^{15}N HSQC Spectrum of EapH2

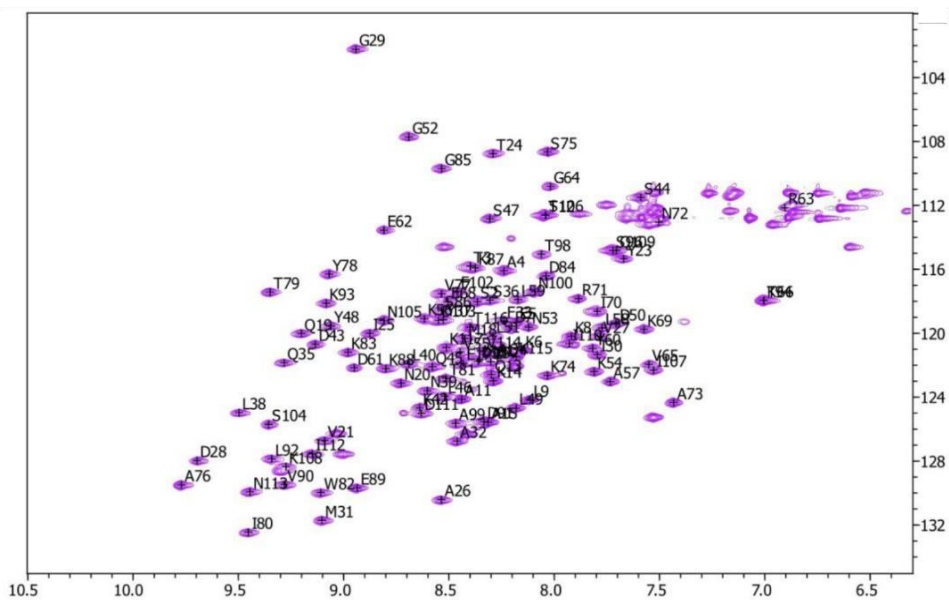


Figure S2. Representative ^1H - ^{15}N HSQC spectra of EapH1 and EapH2. (A) ^1H - ^{15}N HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ -labeled EapH1 recorded at 25 °C on a 600MHz Bruker NMR spectrometer. (B) ^1H - ^{15}N HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ -labeled EapH2 recorded at 25 °C on a 600 MHz Bruker NMR spectrometer. Note that the residue numbering for each protein is based upon the sequence of the recombinant protein following cleavage by TEV protease.

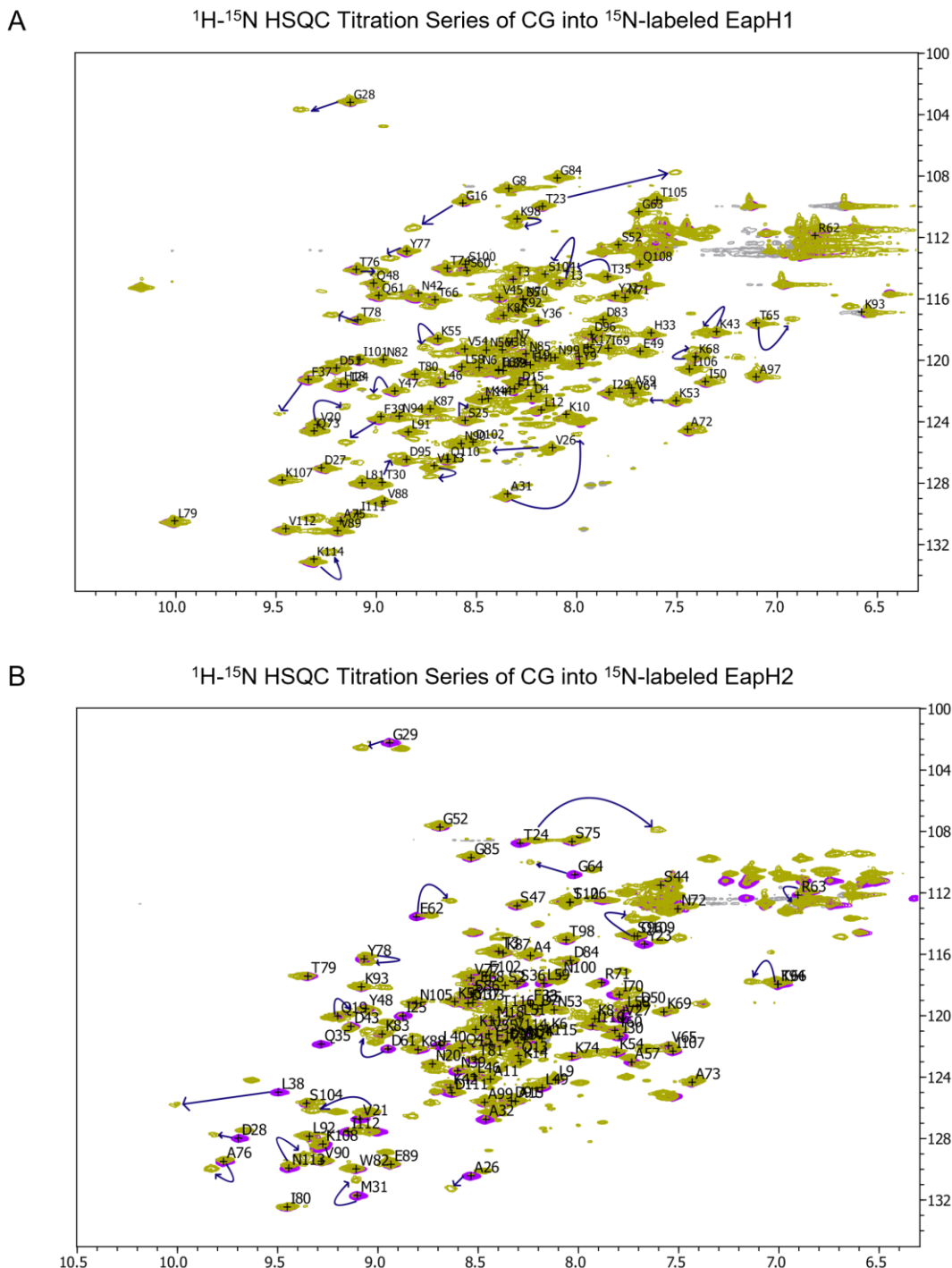
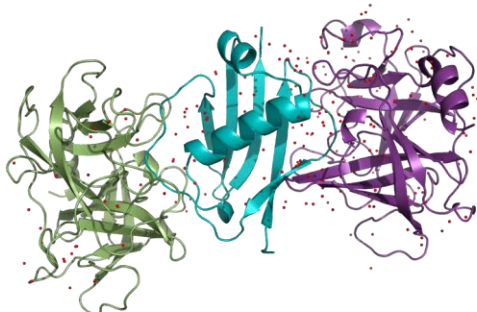


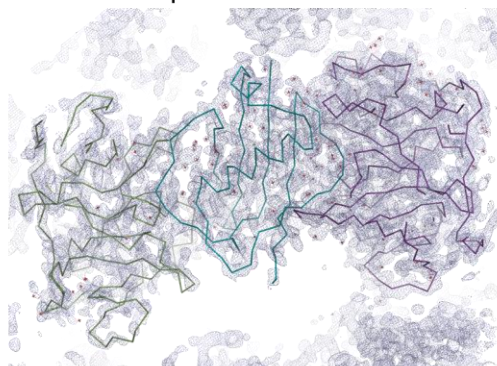
Figure S3. Representative ^1H - ^{15}N HSQC spectra of EapH1 and EapH2 following titration with CG. (A) ^1H - ^{15}N HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ -labeled EapH1 either in the absence (purple) or

Note that the residue numbering for each protein is based upon the sequence of the recombinant protein following cleavage by TEV protease.

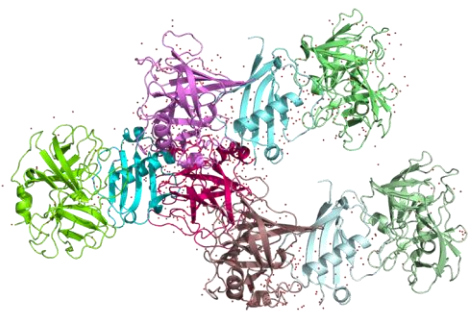
A NE/EapH2/CG Structure – pH 8.5



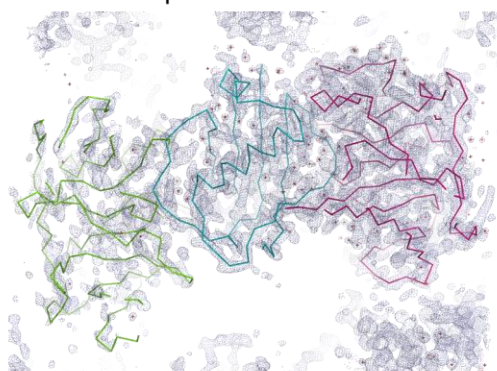
B Model to Map Correlation of Chains A/B/C



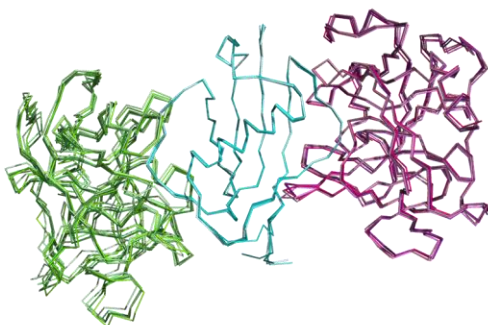
C NE/EapH2/CG Structure – pH 7.5



D Model to Map Correlation of Chains A/B/C



E NE/EapH2/CG Ternary Complex
Superposition of All Models



F NE/EapH2/CG Ternary Complex
B-factor Distribution in Final Models

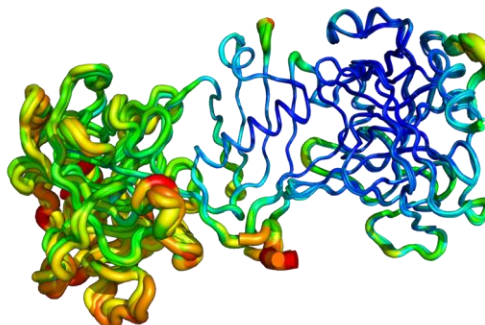


Figure S5. Supporting information concerning crystal structures of EapH2 bound to CG and NE. (A) Final model of the asymmetric unit for CG/EapH2/NE crystallized at pH 8.5 and refined at 1.85 Å limiting resolution. CG (purple), EapH2 (blue), and NE (green) are depicted as ribbon diagrams, while ordered solvent molecules are shown as red asterisks. The N-terminus of EapH2 is positioned centrally at the bottom of the protein as drawn. (B) Model-to-map correlation for the CG/EapH2/NE structure shown in an identical orientation to Panel A. Proteins are depicted as wire traces where only their Cα positions are shown. The 1.85 Å resolution 2Fo-Fc electron density map contoured at 1.2σ is drawn as a grey mesh. (C) Final model of the asymmetric unit for CG/EapH2/NE crystallized at pH 7.5 and refined at 1.80 Å limiting resolution. CG (purples),

EapH2 (blues), and NE (greens) are depicted as ribbon diagrams, while ordered solvent molecules are shown as red asters. Note that there are three unique biological units in this asymmetric unit. (D) Model-to-map correlation for the CG/EapH2/NE structure shown in panel C in an identical orientation to Panel A. For clarity, only the complex represented by chains A, B, and C is shown. Proteins are depicted as wire traces with their C α positions shown. The 1.80 Å resolution 2Fo-Fc electron density map contoured at 1.2 σ is drawn as a grey mesh. (E) Superposition of each model of the CG/EapH2/NE complex from the crystal structures reported here. CG (purples), EapH2 (blues), and NE (greens) proteins are depicted as wire traces with their C α positions shown. (F) Representation of the superimposed CG/EapH2/NE complexes shown in Panel E but colored according to the B-factors of each model. Positions of low B-factors appear thinner and are colored blue, while those with high B-factors appear thicker and are colored red. Note the much higher overall B-factors in the NE proteins when compared to the CG proteins.

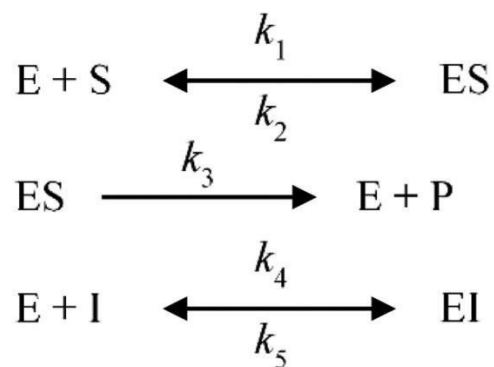


Figure S6. Rapid Equilibrium Competitive Inhibition Mechanism Used for Evaluating Inhibition of CG and NE by EAP Proteins.

Chapter 7 - *S. aureus* Eap Protein is a Bifunctional and Polyvalent Inhibitor of Neutrophil Serine Proteases

Keywords: Protease; Protease Inhibitor; Protein Structure; Innate Immunity; Immune Evasion

Abstract (248 words)

Neutrophils actively survey blood and tissues for pathogenic presence and kill the bacteria through phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs). Neutrophils armed with myeloperoxidase and neutrophil serine proteases (NSPs) mount a formidable defense against pathogens. Certain pathogens, such as *Staphylococcus aureus*, evolved evasion tactics to counter neutrophil attacks due to ongoing host-pathogen interactions. The bacteria produce various proteins including extracellular adherence proteins (Eap) and its homologs, EapH1 and EapH2, to evade killing. Structure-function analysis revealed that EapH1 inhibits both the NSPs, cathepsin-G (CG), and neutrophil elastase (NE), through the globally similar site. However, EapH2 revealed distinct binding sites for both the NSPs, providing it a unique capability to bind both the NSPs simultaneously. Also, Eap domain1 and domain 2 bind CG through the site similar as EapH1 however NE binding site is analogous to EapH2. In this study, we furthered our insight into NSP binding and inhibition by multidomain Eap protein. Crystallography analysis revealed that all four domains can simultaneously bind both the NSPs, similar to EapH2. Since Eap exists as a multidomain protein, we investigated its potential to polyvalently neutralize multiple NSPs concurrently, using SAXS. We discovered that Eap effectively binds four CG molecules or four NE molecules simultaneously in solution. We also tested a two-domain variant of Eap, Eap34, for its ability to fully occupy different NSPs at all its possible sites. HT-SAXS revealed it not only occupies all its sites in solution but also forms higher-order structures when bound to NSPs.

Introduction

Neutrophils, being the most abundant leukocyte in circulation, are the first line of defense against pathogenic microorganisms (1). They patrol the blood and tissue to detect the presence of pathogenic intruders (2–4). They get recruited from the vascular space to the infection site upon detecting the chemoattractants where they neutralize the bacteria through phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs) (1, 5). Neutrophil serine proteases (NSPs) play a central role in all three modes of killing. Following bacterial phagocytosis into the neutrophil phagocytic vacuole neutrophils recruit and fuse primary/azurophilic vacuoles which are armed with antimicrobial proteins like NSPs and MPO, which subsequently kill the microbe (6, 7). Neutrophils can also kill the microbes by releasing their granular content into the extracellular environment by degranulation which can kill the microbe outside of neutrophils (1). Lastly, neutrophils can form NET with the assistance of NE which can entrap and kill bacteria (5). NE plays a central role in NETosis as its release from azurophilic granules into the cytoplasm degrades F-actin, enters the nucleus, hydrolyzes histones, and disrupts chromatin packaging to aid in NET formation (8). NET forms a web-like structure that composes antimicrobial proteins such as histones, NSPs, and MPO at the DNA backbone structure (9). NETs by entrapping the bacteria prevent the dissemination of infection to other than the site of infection (10). Neutrophils, being heavily armed with a diverse array of antimicrobial defense systems, play a central role in fighting against a wide range of pathogens.

Despite presence of a wide range of fighting mechanisms in neutrophils certain species such as *Staphylococcus aureus*, because of concurrent exposure to innate immune arsenals, has evolved an entire array of a highly specific immune evasion strategies to fight back the seemingly invincible host defender (11). These immune evasion strategies provide bacteria to survive and

continue disease pathogenesis despite being attacked by the neutrophils. MPO present in the azurophilic granules performs oxidative killing of bacteria by generating highly toxic hypohalous acid using hydrogen peroxide as a substrate (12). Thereby they are important for killing inside phagosome, in extracellular milieu, and are also a component of NETs. The *S. aureus* secreted protein staphylococcal peroxidase inhibitor (SPIN) acts as a molecular plug to MPO and occludes the substrate access to the active site of MPO thereby blocking the production of hypohalous acids (13). Furthermore, the bacteria secrete a family of proteins known as extracellular adherence proteins (Eap) which can noncovalently bind and inhibit NSPs (14). Inhibiting the serine proteases not only affects the direct bacterial killing in phagosome or in the extracellular milieu but also hampers the formation and effectivity of NETs.

Eap proteins are expressed as a family of three proteins, featuring the single domain known as Eap homologs (EapH1 and EapH2), and the multidomain Eap, which may have four to six repeat domains, varying based on the *Staphylococcus aureus* strain (15–17). Both the Eap homologs and Eap domains have a conserved structural feature adopting a β -grasp fold consisting of a large α -helix superimposed diagonally upon a five-stranded mixed β -sheet (15, 18–21). The structural insights of EapH1 in its NSP bound and unbound state have been explored using protein crystallography. Previous structural studies using CG/EapH1 and NE/EapH1 co-crystal complexes revealed that the protein uses a globally conserved binding mode to bind and inhibit both the serine proteases (14, 22). Site-directed mutagenesis studies signified the role of EapH1-R89 when mutated to methionine. The mutation had a tremendous effect on NE inhibition than on the CG (23). The recent publication has highlighted the NSP binding sites on EapH2. NMR chemical shift perturbation (CSP) studies using EapH1 and EapH2 in the presence of NSPs indicated that EapH2 binds to CG in a way similar to EapH1 however the NE binding site lies away from the NE binding

site of EapH1. Further analysis using x-ray crystallography with CG/EapH2/NE ternary complex bolstered the findings from CSP analysis. These findings show a novel mechanism by which an immune evasion protein can simultaneously bind and inhibit two NSPs (24). Additionally, Eap1 and Eap2 domains identify NE through a mode similar to EapH2 while they identify CG at the site similar to EapH1 (19).

Despite the insights derived from the aforementioned studies, significant questions surrounding the inhibition of neutrophil serine proteases (NSPs) by Eap domain proteins remain unanswered. In this investigation, we explored the structural basis for NSP inhibition by the domains of *Staphylococcus aureus* Eap. While all four Eap domains bind CG through a binding mode similar to EapH1, a distinct binding mode was observed for the binding of NE. Notably, both NSP-binding sites within a single inhibitor were found to be simultaneously occupied, as evidenced by crystal structures of ternary complexes (CG/EapH2/NE) and enzyme assays demonstrating concurrent inhibition of both NSPs. Subsequent investigations on ternary complexes of Eap domain proteins revealed a consistent feature across all Eap domains, demonstrating the ability to simultaneously inhibit both NSPs through distinct binding sites.

A complete inhibition of NE by Eap domain 4 at substoichiometric concentration in the enzyme inhibition study of Eap4/NE prompted a comprehensive investigation using small-angle X-ray scattering (SAXS) and crystallography. SAXS analysis hinted at the possibility of Eap4 uniquely binding two NE simultaneously. We further tested this outcome by crystallography, reaching the same conclusion. The analysis also indicated that the complex exists in a dimer form, suggesting that the full-length Eap molecule can form a higher-order structure upon complexations with NSPs.

Owing to the Eap's composition of tandemly repeating domains and each domain being capable to simultaneously bind CG and NE we sought to explore the possibility if Eap is capable of neutralizing one equivalent of NE and one equivalent of CG by each repeat domain of its sequence in solution. This proposition was examined using SAXS. Previous Mu50 Eap SAXS analysis by Hammel et al. supported the ordered domain assembly and extended conformation of Eap in solution (25). SEC-SAXS analysis with Eap itself and in the presence of CG confirmed its binding capability to CG molecules at all its sites. We performed a similar analysis using NE and revealed the possibility of occupation of all four NE sites on Eap simultaneously. We further extended this study by HT-SAXS analysis of a two-domain Eap variant, Eap34, in its complete NSP occupancy. Analysis revealed simultaneous occupation of these NSPs at their respective sites, along with the formation of a higher-order structure.

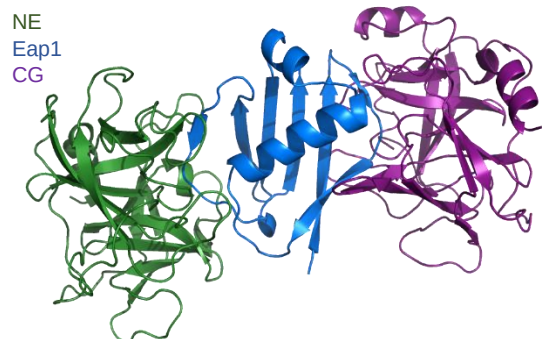
Results

Eap domains are capable of simultaneous inhibition of neutrophil elastase and cathepsin-G

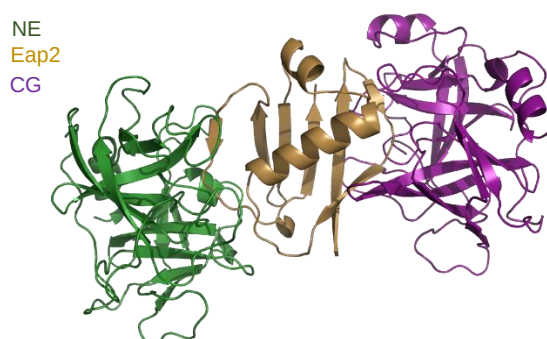
Although EapH1 utilizes the same binding mode to inhibit CG and NE (14, 22), the discovery of two independent NSP-binding sites in Eap1 and Eap2 (19) strongly suggested that these EAP domains may be capable of binding to and inhibiting CG and NE simultaneously. To examine this unexpected possibility, we solved crystal structures of the CG/Eap1/NE and CG/Eap2/NE ternary complexes at 3.63 Å and 3.10 Å resolution, respectively (Fig. 7.1A-B, Fig. S1 & Table 7.1). As with the other protease/inhibitor structures studied here, crystals of CG/Eap1/NE and CG/Eap2/NE each contain multiple copies of their respective complex in the asymmetric unit that do not differ appreciably from each other (Fig. S1A, S1E, S1C & Fig. S1G); we therefore limited our analyses to the complexes represented by Chains A, B, and C in the final

models. We witnessed the possibility of dimerization of the CG/Eap1/NE ternary complex dimerization (Fig. 7.1E) which seems plausible when we analyzed the NSP occupied truncated (Eap 3/4) version of Eap. The representative models of the CG/Eap1/NE and CG/Eap2/NE ternary complexes share clear positional similarities with one another (c.f. Fig. 7.1A & Fig. 7.1B). There is also well-defined electron density encompassing the entire EAP domain in both structures (Fig. S1B & Fig. S1F). Interestingly, we also noticed a far more uniform distribution of B-factors across Eap1 and Eap2 when bound to two proteases simultaneously (Fig. S1D & Fig S1H). This most likely results from protein-protein interactions that limit molecular motions in regions that are otherwise dynamic in the absence of a binding partner, as seen in the structures of the binary complexes (19).

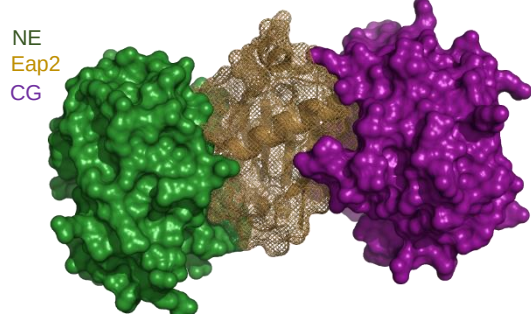
A. CG/Eap1/NE Ternary Complex



B. CG/Eap2/NE Ternary Complex

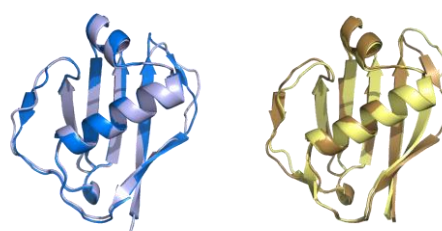


C. Surface Depiction of a Ternary Complex

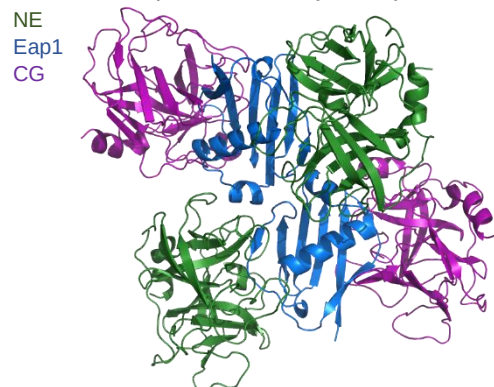


D. EAP Protein Structural Comparison
Apo versus Ternary Complexes

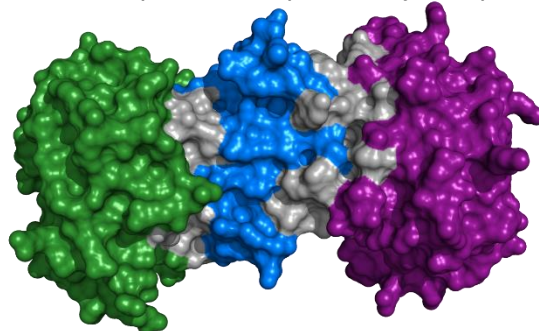
Eap1 (apo)
Eap1 (tern)
Eap2 (apo)
Eap2 (tern)



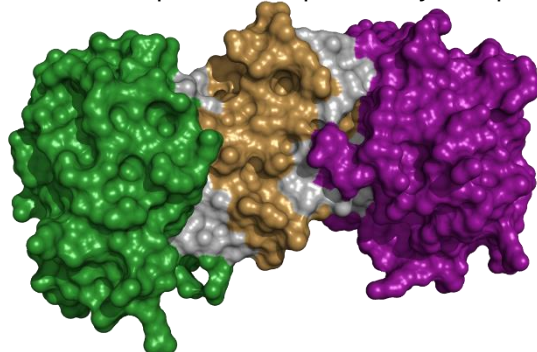
E. CG/Eap1/NE Ternary Complex in Dimer



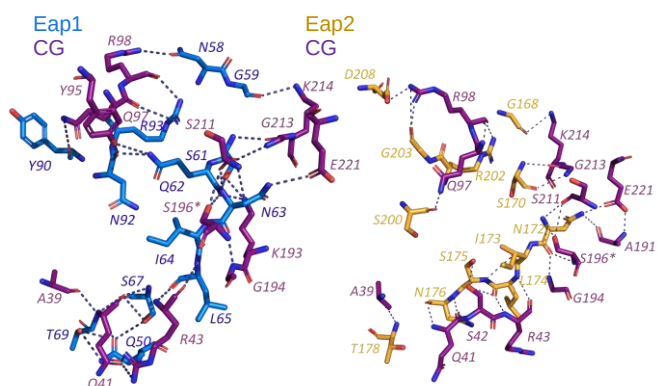
F. Surface Depiction of Eap1 Ternary Complex



G. Surface Depiction of Eap2 Ternary Complex



H. Interactions near CG active site



I. Interactions near NE active site

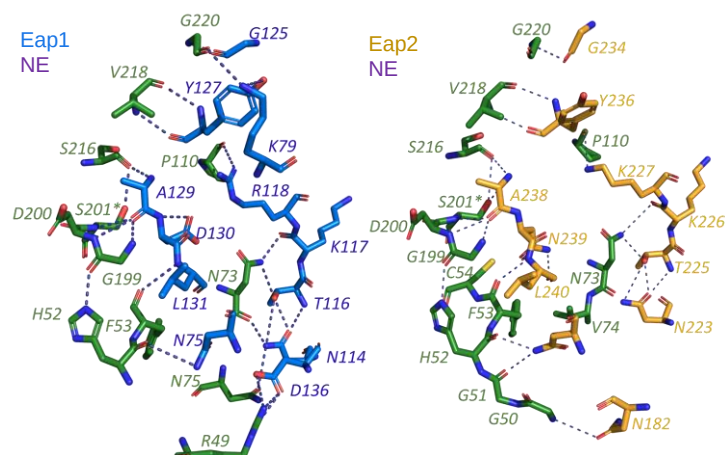


Figure 7.1 Ternary complexes of EAP domain 1 and 2 bound to CG and NE define a structural basis for bifunctional inhibition of Neutrophil Serine Proteases.

(A) Refined model for an individual ternary complex comprised of CG (purple), Eap1 (light blue), and NE (dark green) drawn as a ribbon diagram. Note that Eap1 is colored differently than in previous figures to signify its presence in a ternary complex. (B) Refined model for an individual ternary complex comprised of CG, Eap2 (gold), and NE drawn as a ribbon diagram. As with panel A, Eap2 is colored differently to signify its presence in a ternary complex. (C) Representative image of a CG/EAP/NE ternary complex drawn as a molecular surface. A refined model of the CG/Eap2/NE complex is shown with NE and CG as solid surfaces, while Eap2 is presented as a semi-transparent mesh. Approximately 33% (Eap1) and 36% (Eap2) of the ~6,000 Å² total surface area available in the EAP domain is buried upon formation of such complexes. (D) Comparison of the structures of Eap1 and Eap2 in their free and CG/NE-bound forms. Superpositions of ribbon diagrams for apo Eap1 (lavender) and bound Eap1 (light blue) are shown alongside those of apo Eap2 (light yellow) and bound Eap2 (gold) (15). Note there is little rearrangement within either inhibitor, aside from the loop regions that comprise the protease-binding sites themselves. (E) Refined model for Eap1 ternary complex suggests the presence of a dimeric structure. (F) Surface representation of Eap1 ternary complex to display interactions. CG (purple), Eap1 (light blue), NE (dark green), and interchain interactions (grey) drawn as surface to represent the interactions found between Eap1 and NSPs. (G) Surface representation of Eap2 ternary complex to represent interchain interactions. CG (purple), Eap2 (gold), NE (dark green), and interchain interactions (grey). (H) Representation of interaction of CG with Eap 1 and with Eap 2. Purple (CG), light blue (Eap1), and golden yellow Eap2. (I) Representations of interaction between NE with Eap 1, and with Eap 2. NE (green), light blue (Eap1), and golden yellow Eap2.

Table 7.1 X-ray diffraction data collection, structure solution, and refinement statistics^a

PDB Accession Code	CG/Eap1/NE 8D7I	CG/Eap2/NE 8D7K	CG/Eap3/NE 9ASX	Eap4NE 9ASS	CG/Eap4/NE 9ATK	NE/Eap4/NE 9ATU
Data Collection						
Beamline	APS 22-ID	APS 22-ID	APS 22-ID	APS 22-ID	APS 22-ID	APS 22-ID
Space Group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>F</i> 222	<i>P</i> 1	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Wavelength (Å)	1.000	1.000	1.000	1.000	1.000	1.000
Cell Dimensions a, b, c (Å)	102.23 152.27 296.38	97.59 107.44 105.42	43.97 110.39 116.94	94.62 127.38 148.68	82.21 85.53 89.54	75.82 121.06 147.63
α, β, γ (°)	90.00 90.00 90.00	90.00 91.52 90.00	90.00 90.00 90.00	90.00 90.00 90.00	82.77 85.23 83.60	90.00 90.00 90.00
Resolution (Å)	100.00-3.63	50.00-3.10	50.00-1.95	50.00-1.75	50.00-2.10	50.00-2.05
Wilson B-Factor (Å ²)	84.5	51.0	24.3	23.4	30.8	43.0
Completeness (%)	99.9 (100.0)	99.1 (99.8)	99.5 (98.9)	99.4 (97.8)	90.4 (87.0)	99.7 (99.3)
I / σI	14.3 (4.7)	10.2 (2.8)	10.9 (3.6)	11.5 (1.8)	9.4 (1.7)	20.3 (1.5)
R _{pim}	0.168 (0.768) ^b	0.071 (0.343)	0.111 (0.739)	0.064 (0.641)	0.111 (0.432)	0.029 (0.442)
CC _{1/2}	0.998 (0.938)	0.983 (0.762)	0.965 (0.442)	0.809 (0.580)	0.966 (0.386)	0.990 (0.600)
Redundancy	9.8 (10.18)	4.8 (5.0)	10.4 (6.5)	10.0 (5.5)	2.8 (2.1)	12.8 (9.8)
Refinement						
Resolution (Å)	96.64-3.63	43.83-3.10	40.14-1.95	41.51-1.75	47.89-2.10	41.28-2.05
Number of Reflections	51,218	38,170	41,557	44,362	117,167	79,893
R _{work} / R _{free} (%)	18.6 / 21.7	18.4 / 25.8	19.3 / 22.8	18.9/21.2	17.9 / 23.3	18.5 / 23.7
Atoms Modeled						
Total	25,188	16,684	4,519	2,751	17,992	8,816
Water	0	0	205	246	770	338
Ion	0	0	0	1	0	0
Ramachandran Plot						
Favored/Allowed (%)	93.70 / 5.58	94.27 / 5.31	96.44 / 3.56	98.08 / 1.92	97.23 / 2.72	96.69 / 3.31
B-Factors (Å ²)	95.0	46.0	29.0	32.0	41.0	55.0
R.M.S. Deviations						
Bond Lengths (Å)	0.003	0.009	0.007	0.007	0.008	0.008
Bond Angles (°)	0.69	1.10	0.86	0.80	0.91	0.89

^aValues in parentheses are for the highest-resolution shell.^bValues represent R_{merge} as calculated by xScale.

We found the structures of each ternary complex to be an amalgamation of the respective binary complexes. In both cases, CG occupies the EapH1-like inhibitory site seen in the CG/Eap1 and CG/Eap2 structures (19), while NE occupies the newly identified inhibitory site seen in the NE/Eap1 and NE/Eap2 structures (19). The nature and extent of the interactions formed at the individual interfaces are comparable to those of the binary complexes described in earlier sections. This includes the presence of hydrogen bonds between the EAP domains and groups derived from the catalytic serine of both proteases (Fig. 7.1H & I). The shape complementary coefficients of the individual interfaces differ by only 1% on average with those seen in the binary complex structures. Similarly, the total buried surface area in each complex is comparable to the sum of the respective binary complexes. CG/Eap1/NE buries a total of 2,028 Å² (Fig. 7.1F), which is ~5% greater than

the sum of CG/Eap1 and NE/Eap1 (i.e. 1,937 Å²), while CG/Eap2/NE buries a total of 2,128 Å² (Fig. 7.1G), which is ~10% greater than the sum of CG/Eap2 and NE/Eap2 (i.e. 1,931 Å²). These buried surface area values correspond to ~33% and ~36% of the total available surface area in Eap1 and Eap2, which can be inferred by examining a representative complex rendered as a molecular surface (Fig. 7.1C, F & G). Nevertheless, superposition of the EAP domain structures from the ternary complexes with their unbound forms reveals significant positional rearrangement within both the CG and NE-binding sites (Fig. 7.1D). This results in what appear to be new stretches of β -strand in both NSP-binding sites, which arise via formation of hydrogen bonds between mainchain-derived groups from the inhibitor and protease (19). Presumably, such rearrangements are facilitated by the inherent flexibility of these regions in the EAP domains, as judged by their B-factors in the absence of a binding partner (19).

Furthermore, we also explored the possibility of ternary complex formation with third and fourth domain of Eap. CG/Eap3/NE crystal structure complex with 1.86 Å limiting resolution consisting of 1 copy of the complex in ASU identified that Eap3 identifies CG at the binding site similar to other Eap domains i.e. EapH1-like. However, NE occupied the Eap3 at the site similar to EapH2 (Fig 7.2A). The NSP interaction sites at Eap3 are found to be similar to commonly found interactions at other Eap domains including the hydrogen bonds with catalytic serine at CG and NE (Fig. 7.2E). The shape complementarity coefficient for CG/Eap3 interface was found to be 0.705 while 0.718 for NE/Eap3 interface. The total buried surface area calculated from PISA for CG/Eap3/NE is found to be 2060 Å² which accounts for 33% for the total available surface area for Eap3 (Fig. 7.2C).

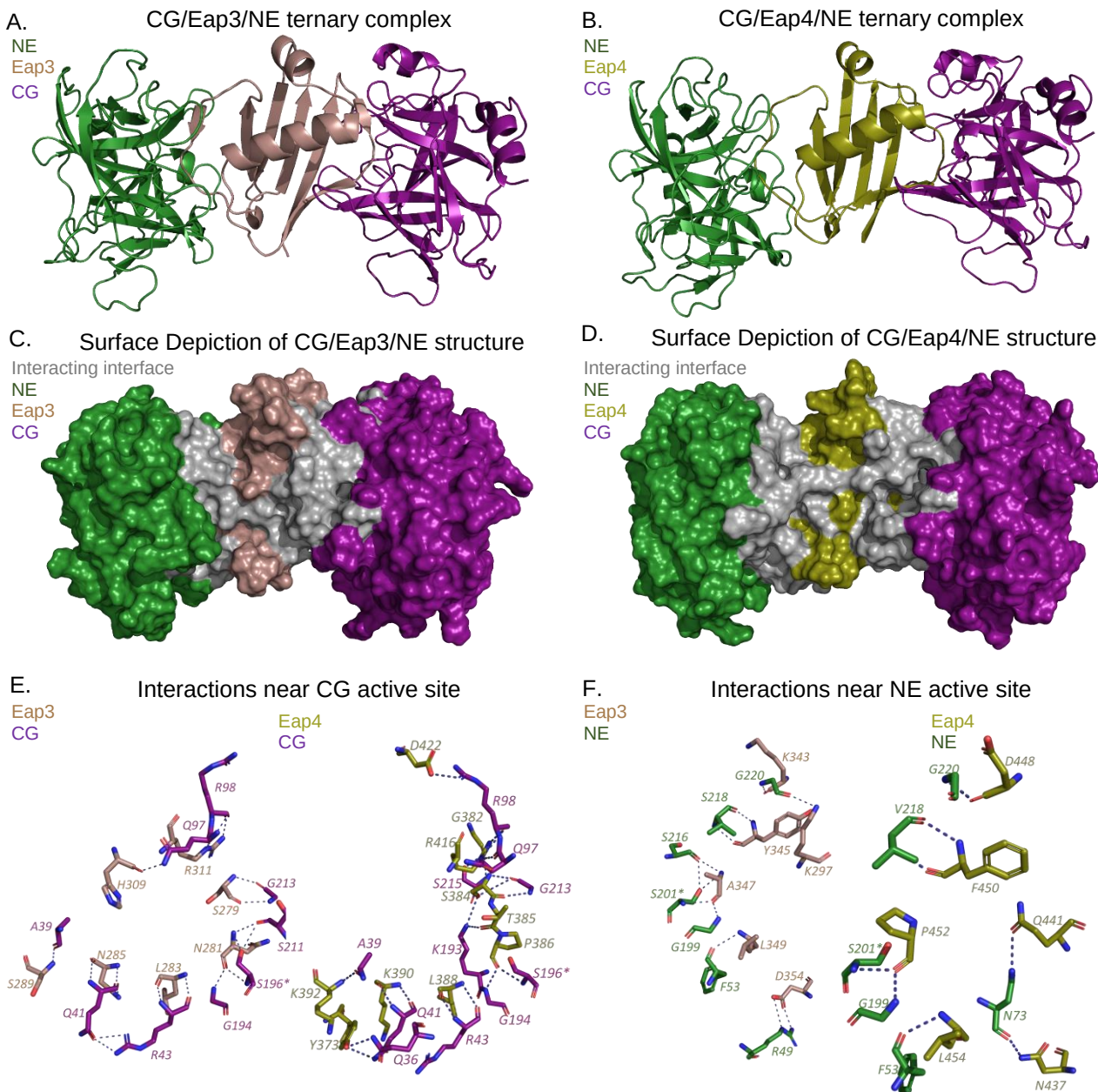


Figure 7.2 Ternary complexes of EAP domain 3 and domain 4 bound to CG and NE display a similar mode of NSP inhibition as Eap domain 1 and domain 2.

(A) Cartoon representation of refined model for Eap3 ternary complex comprised of CG (purple), Eap3 (wheat), and NE (dark green). (B) Refined model for Eap4 ternary complex comprised of CG (purple, chain I), Eap4 (deep olive, chain C), and NE (green, chain A) drawn as a ribbon diagram. (C) Representation of CG/Eap3/NE ternary complex drawn as a molecular surface. A refined model of the CG/Eap2/NE complex is shown with NE and CG as solid surfaces, while Eap2 is presented as a semi-transparent mesh. Approximately 33% (Eap1) and 36% (Eap2) of the ~6,000 Å² total surface area available in the EAP domain is buried upon formation of such complexes. (D) Comparison of the structures of Eap1 and Eap2 in their free and CG/NE-bound forms. Superpositions of ribbon diagrams for apo Eap1 (lavender) and bound Eap1 (light blue) are shown

alongside those of apo Eap2 (light yellow) and bound Eap2 (gold) (15). Note there is little rearrangement within either inhibitor, aside from the loop regions that comprise the protease-binding sites themselves. (E) Refined model for Eap1 ternary complex suggests the presence of a dimeric structure. (F) Surface representation of Eap1 ternary complex to display interactions. CG (purple), Eap1 (light blue), NE (dark green), and interchain interactions (grey) drawn as surface to represent the interactions found between Eap1 and NSPs. (G) Surface representation of Eap2 ternary complex to represent interchain interactions. CG (purple), Eap2 (gold), NE (dark green), and interchain interactions (grey).

CG/Eap4/NE complex crystal structure with 2.11 Å limiting resolution consisting of 4 ternary complexes in the ASU (Fig. S2A). Close inspection of the ASU contents indicates the possibility that the complexes might be forming a dimer through the C-terminal region of Eap4 (Fig. S2C). These complexes do not differ significantly from one another (Fig. S2B), so we limited our analysis to the complex represented by chain ACI (Fig. 7.2B). Eap4, like other Eap domains, binds to CG and NE i.e. it recognizes CG like EapH1 and NE through the site similar to EapH2. The nature of interactions present at both the NSP sites are comparable with other Eap domains including H-bond near the NSP catalytic serine (Fig 7.2E & 7.2F). The shape complementarity coefficient for CG/Eap4 interface was found to be 0.803 while 0.706 for NE/Eap4 interface. The total buried surface area calculated from PISA for CG/Eap4/NE is found to be 2016 Å² which accounts for 32% of the total available surface area for Eap4 (Fig 7.2D).

The crystal structures of Eap domains bound to NSPs show that a single EAP domain can occlude the active site of both NSPs. However, we sought additional information to determine whether such complexes still blocked NSP activity in solution. To probe the ability of Eap1 and Eap2 to simultaneously inhibit CG and NE, we compared the progress curves and extent of inhibition of reactions containing both NSP enzymes, their substrates, and an Eap protein inhibitor with reactions consisting of only one NSP, both protease substrates, and the same Eap protein inhibitor (Fig. 7.3 & Fig. S3). To facilitate interpretation of the data, we employed a colorimetric

substrate of CG (i.e. SA₂PF-pNA) and a fluorescent substrate of NE (i.e. MSA₂PV-AMC). We found that the extent of CG inhibition upon concurrent exposure of the enzyme to its substrate and Eap1 is unchanged by the presence of NE (Fig. 7.3A); similarly, we found that NE inhibition is unchanged upon concurrent exposure of the enzyme to its substrate and Eap1 by the presence of CG (Fig. 7.3B). We observed altogether similar results in analogous set of experiments where Eap2 was substituted for Eap1 in reactions that monitor CG (Fig. 7.3C) and NE activity (Fig. 7.3D), respectively. We also performed complementary studies where either CG or NE was pre-incubated with either Eap1 (Fig. S3A & S3B) or Eap2 (Fig. S3C & S3D) prior to exposure to the other components. Again, we found that neither Eap1 nor Eap2 loses inhibitory potency in the presence of an additional protease. By contrast, when we conducted a similar experiment with EapH1, we found that including NE in the reaction restored CG activity (Fig. S3E). We did not observe recovery of NE activity in the corresponding experiment (Fig. S3F). However, the apparent affinity of EapH1 for NE is ~2,000-fold higher than for CG (24) , strongly implies that EapH1 will continue to be associated with NE under these circumstances. These results provide evidence that Eap1 and Eap2 can simultaneously bind and inhibit both enzymes in solution, but that EapH1 cannot. When considered alongside the structures presented above, we suggest that Eap1 and Eap2 define mechanism for bifunctional inhibition of NSPs.

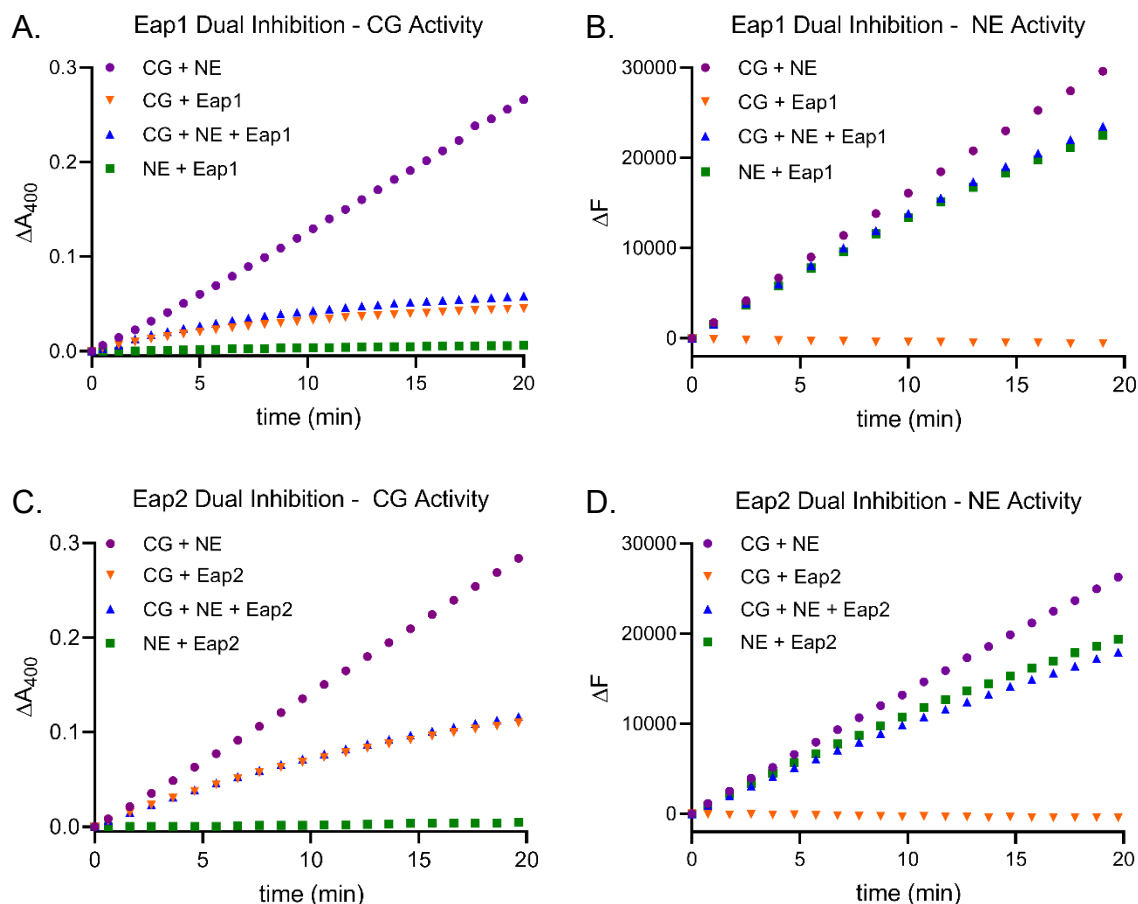


Figure 7.3 Simultaneous inhibition of Cathepsin-G and Neutrophil Elastase by Eap1 and Eap2.

(A) Simultaneous inhibition of CG and NE by Eap1 followed by SA₂PF-pNA turnover by CG. (●) Reaction consists of 7.5 nM CG, 7.6 nM NE, 0 nM Eap1. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 9.4 nM Eap1. (▲) Reaction consists of 7.5 nM CG, 7.6 nM NE, 9.4 nM Eap1. (■) Reaction consists of 0 nM CG, 7.6 nM NE, 9.4 nM Eap1. (B) Simultaneous inhibition of CG and NE by Eap1 followed by MSA₂PV-AMC turnover by NE. (●) Reaction consists of 7.5 nM CG, 7.6 nM NE, 0 nM Eap1. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 9.4 nM Eap1. (▲) Reaction consists of 7.5 nM CG, 7.6 nM NE, 9.4 nM Eap1. (■) Reaction consists of 0 nM CG, 7.6 nM NE, 9.4 nM Eap1. (C) Simultaneous inhibition of CG and NE by Eap2 followed by SA₂PF-pNA turnover by CG. (●) Reaction consists of 8.2 nM CG, 7.3 nM NE, 0 nM Eap2. (▼) Reaction consists of 8.2 nM CG, 0 nM NE, 10.3 nM Eap2. (▲) Reaction consists of 8.2 nM CG, 7.3 nM NE, 10.3 nM Eap2. (■) Reaction consists of 0 nM CG, 7.3 nM NE, 10.3 nM Eap2. (D) Simultaneous inhibition of CG and NE by Eap2 followed by MSA₂PV-AMC turnover by NE. (●) Reaction consists of 8.2 nM CG, 7.3 nM NE, 0 nM Eap2. (▼) Reaction consists of 8.2 nM CG, 0 nM NE, 10.3 nM Eap2. (▲) Reaction consists of 8.2 nM CG, 7.3 nM NE, 10.3 nM Eap2. (■) Reaction consists of 0 nM CG, 7.3 nM NE, 10.3 nM Eap2. Substrate concentrations in all reactions were 1.175 mM SA₂PF-pNA and 170 μM MSA₂PV-AMC.

Eap domain 4 can simultaneously bind two NE

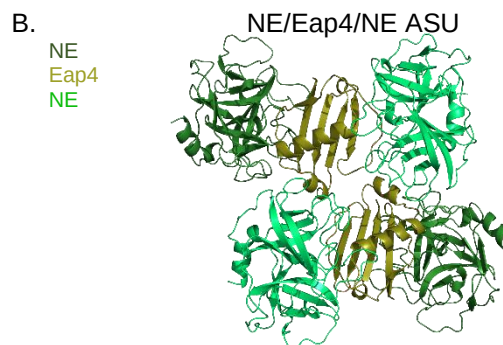
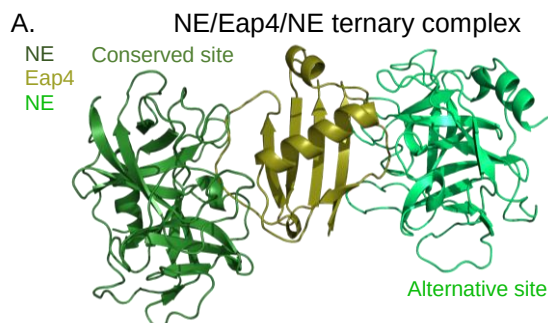
Eap domain 4 exclusively demonstrated the ability to fully inhibit NE even at sub-stoichiometric concentrations, suggesting the potential presence of multiple NE binding sites on Eap4. To unravel these complexities in the enzyme kinetic data, we investigated the possibility of multiple sites for NE at Eap 4 by SAXS approach. Sec-SAXS analysis was conducted with Eap4 in the presence of 2 molar and 3 molar excesses of NE. Both analyses yielded similar R_g , MW estimation, and GNOM D_{max} , indicating the formation of a complex in the presence of a two-fold excess of NE (Table 7.2). Further narrowing our focus to a 2-molar excess of NE, Eap4/2NE exhibited an R_g value of 36.82 (± 0.25), which is higher compared to monomeric EapH2/CG/NE (28.31 ± 0.23). Additionally, the GNOM D_{max} value for Eap4/2NE (110 Å) exceeded that of CG/EapH2/NE (94 Å), as did the calculated molecular weight. The molecular weight derived from the SAXS profile closely aligns with the Eap4/2NE complex in its dimeric state (Fig 7.4E & Table 7.2).

To gain deeper insights into this unusual characteristic, we crystallized and determined the structure of the Eap4/2NE complex with a resolution limit of 2.05 Å (Fig. 7.4A). The ASU content analysis revealed a singular instance of dimerized Eap4/2NE (Fig 7.4B). Upon overlaying/aligning both complexes using PyMol with an RMSD of 0.806, chain ABC was employed to represent and illustrate interactions. Eap4 binds to one NE at a site previously identified for all modular Eap domains (called conserved site), while the other NE in the ternary complex binds to the site resembling EapH1 (called alternative site) (Fig 7.4A). Eap4 interacts with the catalytic serine of both NEs at its binding sites (Fig 7.4C & D). The nature and extent of interactions at both binding sites are comparable to NE interactions with EapH1 and EapH2. The presence of an additional NE binding site at Eap4 sets it apart among other Eap domain proteins. Interestingly, the complex

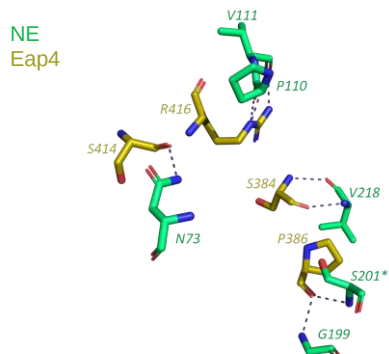
forms a dimer through the interactions at C-terminal region of Eap4, supporting the feature observed from SEC-SAXS (Fig 7.4B, S2D). Comparison of the theoretical SAXS profile of the Eap4/2NE dimer crystal structure with the experimental SAXS profile resulted in an X^2 of 1.41, while fitting was less satisfactory with the monomeric complex (Fig. 7.4E). DENSS calculated density map fits well to the crystal structure of NE/Eap4/NE dimer (Fig. 7.4F). A larger Dmax of the NE/Eap4/NE and an altogether different Kratky plot, compared to monomeric CG/EapH2/NE (Fig. 7.4G & H), further supports the notion of a dimeric presence in the solution (Fig. 7.4I & J). We also crystallized Eap4/NE binary complex which shows that NE preferentially binds the conserved site (Fig. S2E) only in the presence of excess NE; it also occupies the alternative site. Analysis of the crystal structure indicates similarities to the EapH1/NE cocrystal structure, particularly in the interactions surrounding NE's catalytic serine (Fig. S2F & S2G) (14). Moreover, the B-factor distribution of Eap4/NE closely resembles that of EapH1/NE and other Eap/NE binary complexes (Fig. S2H) (14, 19). Notably, the B-factor distribution in the Eap4/2NE ternary complex shares similar features, higher B-factor at alternate NE binding site, as that of CG/EapH2/NE ternary complex (Fig. S2I) (24).

Table 7.2 Overview of SAXS derived parameters

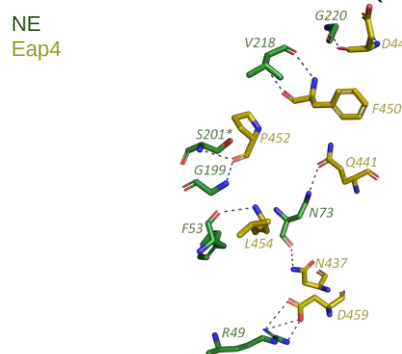
Protein sample	Structural Parameters			Molecular Weight (kDa)				Experimental technique
	Guinier Rg (Å)	Guinier I(0)	GNOM Dmax (Å)	Vp	Vc	S&S	Bayes	
EapH2	19.1 ± 0.08	23.44 ± 0.07	79.0	12.4	13.9	15.6	13.4	SEC-SAXS
CG	18.64 ± 0.11	15.32 ± 0.06	69.0	22.1	19.9	20.0	20.6	SEC-SAXS
CG/EapH2/NE	28.31 ± 0.23	56.02 ± 0.21	94.0	58.4	49.0	52.5	53.1	SEC-SAXS
NE/Eap4/NE	36.82 ± 0.25	81.96 ± 0.47	110.0	123.4	111.4	117.7	113.7	SEC-SAXS
Eap	40.3 ± 0.59	53.14 ± 0.72	153.0	59.9	47.1	-	45.7	SEC-SAXS
Eap/4CG	50.32 ± 0.89	146 ± 2.26	202.0	138.5	117.3	-	124.5	SEC-SAXS
Eap/4NE	41.24 ± 1.58	15.18 ± 0.51	192.0	126.3	103.4	147.8	97.5	SEC-SAXS
Eap34	24.73 ± 0.33	18.65 ± 0.18	85.0	22.1	19.7	21.4	21.2	SEC-SAXS
Eap34/2CG/2NE	53.67 ± 1.13	134 ± 1.18	197.0	231.8	210.5	232.7	208.0	HT-SAXS



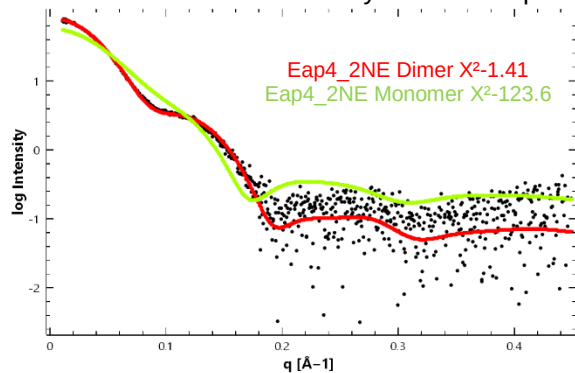
C. Interactions at NE active site (Alternative site)



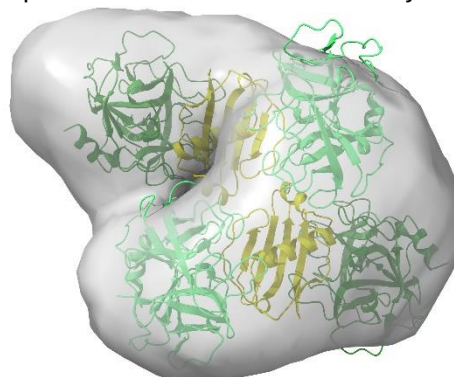
D. Interactions at NE active site (Conserved site)



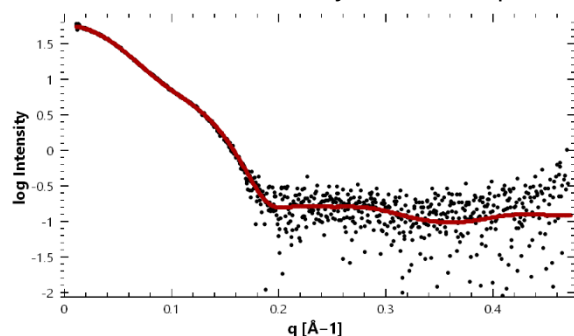
E. Sec-SAXS analysis of NE/Eap4/NE



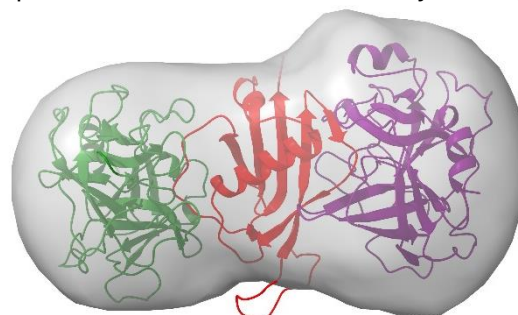
F. NE/Eap4/NE in DENSS electron density envelope



G. Sec-SAXS analysis of CG/EapH2/NE



H. CG/EapH2/NE in DENSS electron density envelope



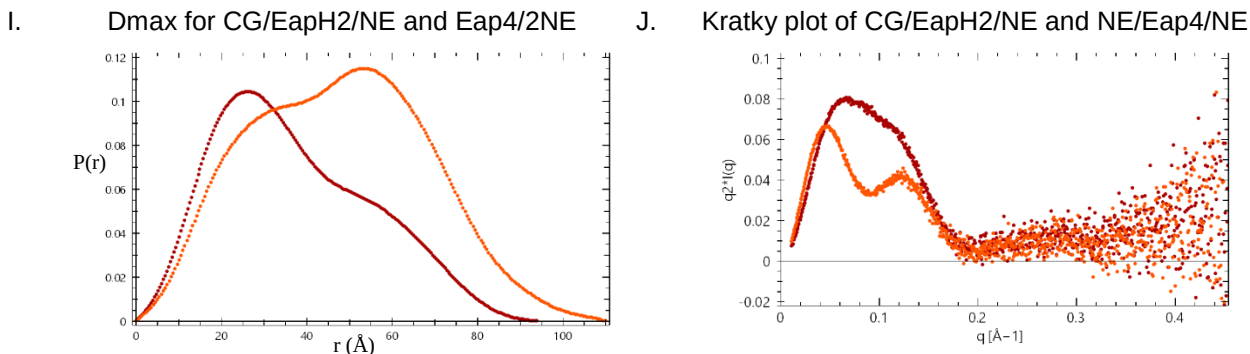


Figure 7.4 EAP domain 4 can simultaneously neutralize two NE through both its NSP binding sites.

(A) Cartoon representation of Eap 4 bound to two NE, one is analogous to NE recognition site at other Eap domains, conserved site (Green) other NE recognition site at Eap4 is analogous to EapH1, alternative site (Lime Green), Eap 4 is represented in sand color. (B) Cartoon representation of Eap 4 bound to two NE showing dimerization of the complex at the C-terminus of Eap 4, NE at conserved site (Green), NE at alternative site (Lime Green). (C & D) Interactions near the NE active sites as occupied by Eap 4, Eap4 (Sand), conserved site (Green), alternative site (Lime Green). (E) Fits between experimental profile (Black), Eap 4 bound to two NE, monomer (Light Green) and Eap 4 bound to two NE, Dimer (Red). (F) Eap 4 bound to two NE (Dimer) docked into the electron density envelope. (G) Fits between the Sec-SAXS profile (Black) and CG/EapH2/NE crystal structure (Ruby red). (H) CG/EapH2/NE crystal structure docked into electron density envelope. (I) $P(r)$ function for CG/EapH2/NE (Ruby red) and Eap4/2NE (Orange). (J) Kratky plot CG/EapH2/NE (Ruby red) and Eap4/2NE (Orange).

Eap is a polyvalent and bifunctional inhibitor of NSPs

We investigated the solution structure of Eap and its truncated versions in unbound and NSP-bound forms using SAXS. Eap eluted as a single asymmetric peak in SEC-SAXS, indicating the absence of aggregation. Guinier analysis provided an R_g value of $40.3 \text{ Å} (\pm 0.59)$. Molecular weight estimation from the SAXS profile corresponded to the monomeric form of Eap. Utilizing the Dmax (GNOM) estimation of 153 Å (Table 7.2), we generated an electron density envelope using DENSS (Fig. 7.5A & G), consistent with previous findings by Hammel et al (25). For ensemble generation and model optimization, we employed multiFOXS. Given the presence of four tandem repeat domains in Eap, we utilized the comparative modeler program in ChimeraX to

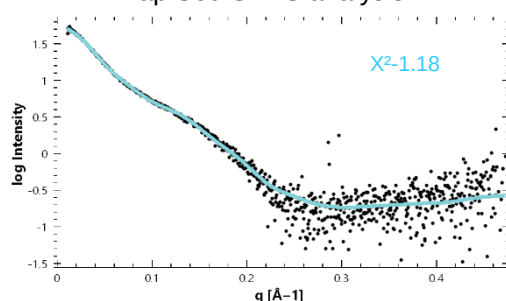
connect these domains. Hinge regions linking the domains and flexible regions were allowed flexibility during conformational sampling and multi-state structural modeling by multiFOXS. The resulting models for Eap attained X^2 values of 1.18 for one state and 1.11 for two states (Fig. 7.5A and 5B).

We also investigated Eap in its 4CG-occupied state using SEC-SAXS (Fig. 7.5C). Similar to Eap, Eap-4CG displayed a single asymmetric peak, indicating the absence of aggregation or oligomerization, with an R_g of 50.32 Å (± 0.89), expectedly higher than that of Eap itself. Molecular weight estimation from the SAXS profile yielded a slightly lower value compared to the anticipated MW of ~166 kDa. To address this, we conducted and analyzed SEC-SAXS of hCG. The molecular weight estimation from the SAXS profile for hCG, calculated from a single asymmetric peak, was approximately 8 kDa less than the expected MW (29 kDa) (Fig. 7.5H), elucidating the observed discrepancy in the MW estimation from the Eap-4CG SEC-SAXS profile. The GNOM D_{max} estimation (202 Å) from the Eap-4CG SEC-SAXS profile indicates that the complex is in a highly extended state (Table 7.2). For ensemble generation and model optimization using multiFOXS, we utilized the CG/Eap domain/NE crystal structure. We removed the NE and linked the domains with linker residues from the sequence, employing the comparative modeler tool available in ChimeraX. The resulting model achieved X^2 values of 2.09 for one state and 1.34 for two states (Fig. 7.5C & D).

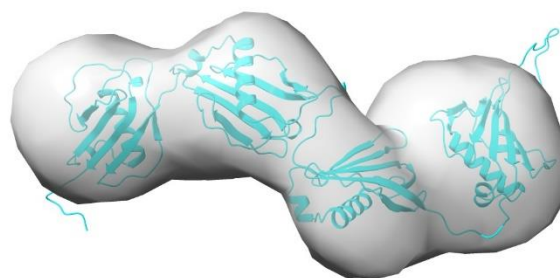
SEC-SAXS analysis of Eap complexed with 4NE resulted in a broad peak, indicating multiple states across the elution. The Guinier approximation yielded an R_g of 41.24 Å (± 1.58), which is 9.08 Å less than that of Eap complexed with CG, suggesting a relatively compact particle. The calculated GNOM D_{max} for Eap/4NE was 192.0 Å, also lesser than its CG counterpart (Fig. 7.5G). This implies that Eap adopts a more extended conformation when in complex with CG

rather than with NE (Fig. 7.5D & F). Molecular weight estimation from the SAXS profile again resulted in a lower value (~ 117 kDa) compared to the expected (~ 166 kDa) (Table 7.2). Although SEC-SAXS data for NE itself was not available, the superimposition of CG and NE crystal structures with a 0.647 Å RMSD suggests that the SAXS behavior for NE will likely mirror that of CG, providing an explanation for the lower molecular estimation for Eap4NE by SAXS. The modeled structure achieved χ^2 values of 0.74 for one state and 0.66 for two states (Fig. 7.5E & F).

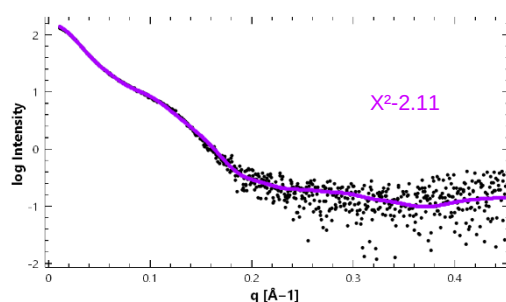
A. Eap Sec-SAXS analysis



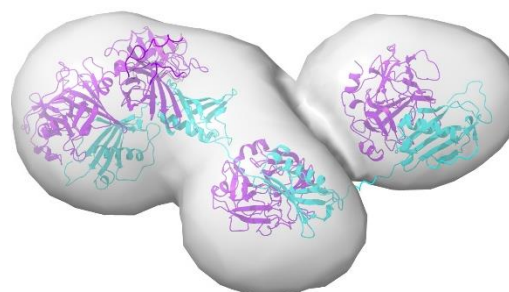
B. Eap model in electron density envelope



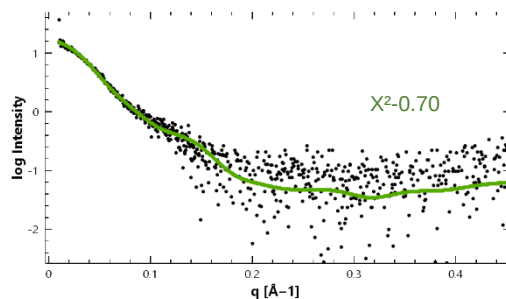
C. Eap/4CG Sec-SAXS analysis



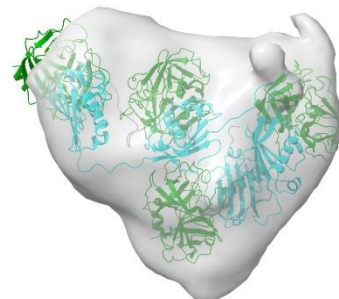
D. Eap/4CG model in electron density envelope



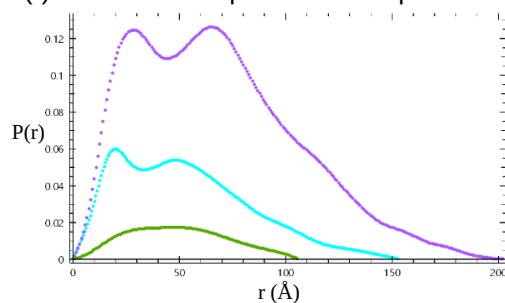
E. Eap/4NE Sec-SAXS analysis



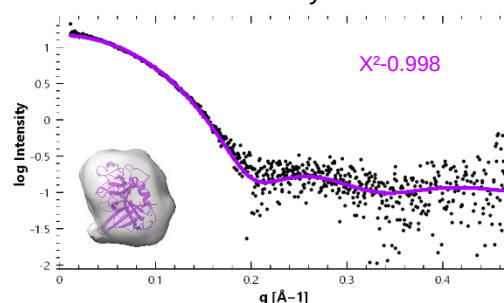
F. Eap/4NE model in electron density envelope



G. P(r) function for Eap in NSP occupied state



H. Sec-SAXS analysis of hCG



I. Kratky plot of Eap in NSP occupied state

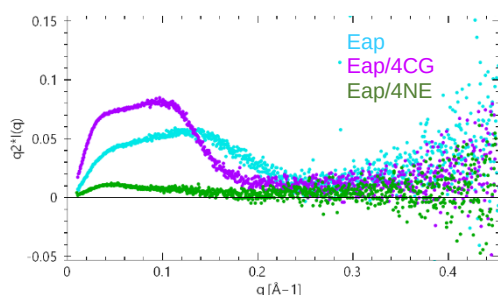


Figure 7.5 Sec-SAXS analysis of Eap and its complexes with NSPs.

(A) Fits between experimental SAXS profile (Black) and 1 state Eap model (Cyan). (B) 1-state Eap model docked into electron density envelope. (C) Fit between the experimental SAXS profile (Black) and 1-state Eap with its all four sites occupied by CG (Green). (D) 1-state Eap (Cyan) fully occupied to CG (Green) docked into electron density envelope. (E) Fits between experimental SAXS profile (Black) and Eap fully occupied with NE (Green). (F) Eap (Cyan) completely occupied with NE (Green) docked into electron density envelope. (G) $P(r)$ function of Eap occupied by NSPs. (H) Fits between experimental (Black) and CG crystal structure model (Purple). CG crystal structure docked into electron density envelope shown in the bottom left.

HT-SAXS experiments were conducted on Eap3/4 with a 2-fold excess of CG and NE at concentrations of 5, 2.5, and 1.25 mg/ml. As there was no observed aggregation or discernible difference in the SAXS profiles, our analysis focused on the 5 mg/ml concentration (Fig. 7.6E). The Guinier approximation yielded an R_g of 48.84 Å (± 0.19), and the calculated GNOM D_{max} of 195 Å appeared reasonable for this complex (Fig. 7.6F & Table 7.2). The structure was modeled into the SAXS profile using multiFOXS, followed by BilboMD achieving X^2 0.33 for one state and 0.12 two states (Fig. 7.6C & D). This result supports our hypothesis of Eap inhibiting multiple NSPs simultaneously in solution. The crystal structure analysis anticipated the formation of a dimer, and indeed, dimerization occurred only upon complexation with NSPs.

Analysis of Eap3/4 in the absence of any NSPs revealed an R_g of 24.73 Å (± 0.33) and an estimated molecular weight of ~21 kDa (Fig. 7.6A & B). The estimated molecular weight and D_{max} of 85 Å closely matched the monomeric form of Eap3/4 (Table 7.2). Utilizing the crystal

structure from the Eap3 and 4 ternary complexes, we calculated the solution structure by multiFOXS, successfully modeling the structure with an X^2 of 0.81.

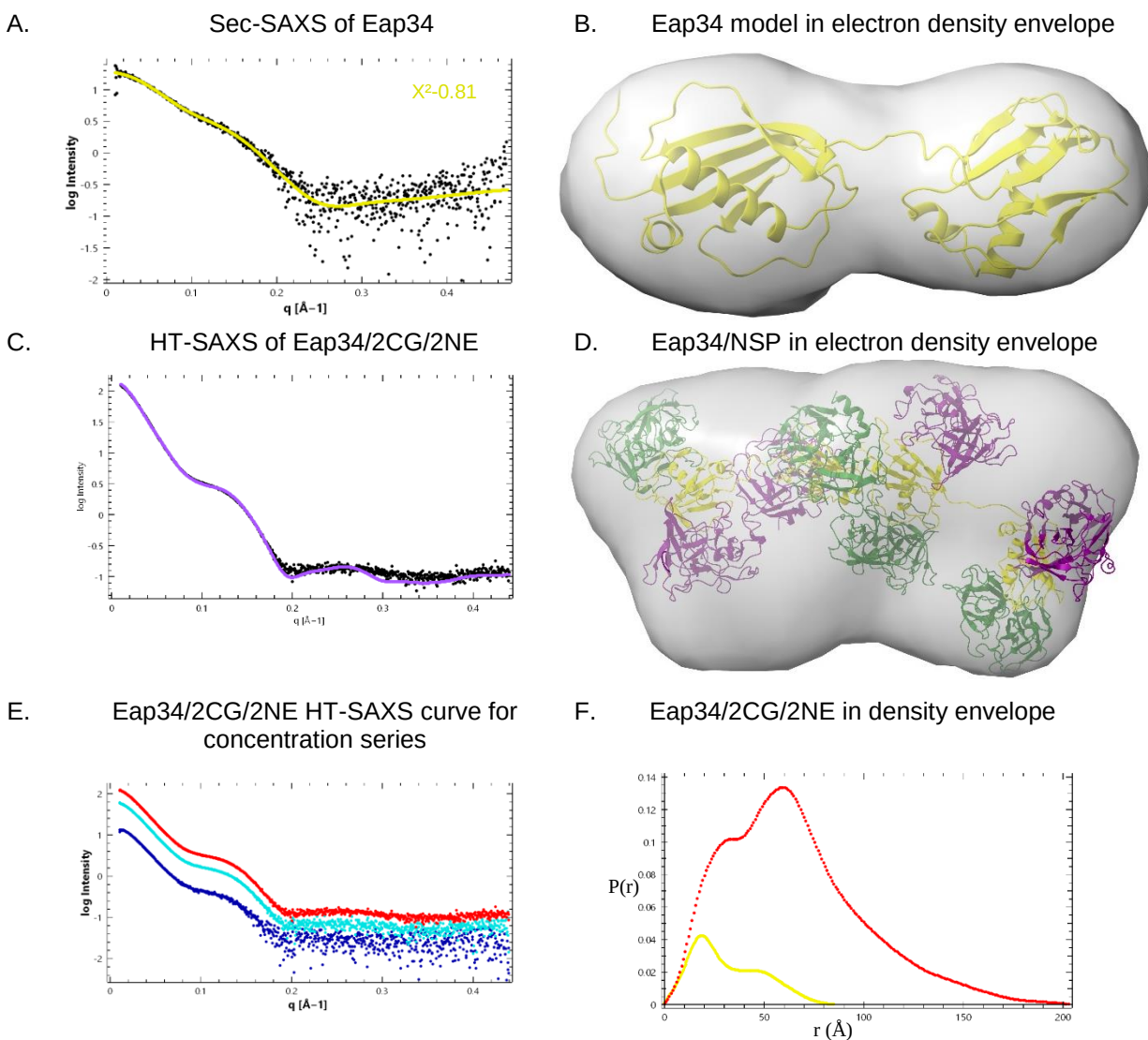


Figure 7.6 SAXS analysis of Eap34 bound to NSPs.

(A) Fitting of experimental SAXS profile (Black) and Eap3/4 model (Yellow). (B) 1-state model of Eap3/4 docked into the electron density map. (C) Fitting of experimental SAXS profile of Eap34 bound to CG and NE (Black) to its single state model (Purple). Docking of Eap34 bound to CG and NE model into the electron density map. (E) Eap3/4 bound to CG and NE at concentrations of 5 (Red), 2.5 (Cyan), and 1.25 (Dark blue) mg/ml. They show no discernible differences. (F) P(r) function denoting Dmax of the particle, Eap34 (yellow) and Eap34/2CG/2NE (Red).

Discussion

Discovery of NSP inhibition by Eap proteins has expanded our understanding of the mechanism of staphylococcal innate immune evasion. EapH1 served as a classic example in understanding the inhibition mechanism as it was the first to be explored well using structure-function analysis. These analyses concluded that the EapH1 uses a globally similar mode to inhibit both NE and CG (14). Our recent finding has shed light on NSP inhibition by Eap domain 1 and 2 by structure/function characterization, indicating that both the domains recognize CG through the site similar to EapH1 however the NE binding site is completely distinct, and lies on the opposite site of CG binding. A similar contrast in the inhibition mode of EapH1 and EapH2 was indicated using site-directed mutagenesis. EapH1 R89M had a major impact on NE inhibition however EapH2 R90M mutagenesis has no visible impact on NE inhibition. These observations hint at a different binding site for NE by EapH2 (26). Our recent structural explorations using ternary complexes of CG/EapH2/NE showed that indeed EapH2 binds to NE at a site different than that of EapH1. These structures not only provided the first empirical description of an alternative NSP-binding mode in EAP domain proteins, but they also raised the possibility that certain EAP domain proteins might be capable of forming ternary complexes with CG and NE. Consistent with that premise, we determined crystal structures of ternary complexes of Eap domains with CG and NE (Fig.7.1 & Fig. 7.3) and further showed that such complexes inhibit the activity of both NSPs simultaneously (Fig. 7.2 & Fig. S2). Having all these explorations in mind it can be inferred that EapH1 strangely inhibits both the NSPs using the same site however EapH2 and all Eap domains use a distinct binding mode to inhibit these NSPs. This peculiarity of EapH1 prompts us to shift the paradigm of NSP inhibition from EapH1 to EapH2, as all other Eap domain proteins behave more like EapH2 than EapH1.

The crystallography and inhibition studies provided a detailed understanding and solid evidence supporting simultaneous NSP inhibition by Eap domain proteins. However, the possibility of a polyvalent NSP inhibition by multidomain Eap protein emerged from SAXS analysis. Previous SAXS analysis of multidomain Eap protein showed the Eap adopts an extended but structured conformation in solution (25). The outcomes presented here for Eap analysis by Sec-SAXS show that Eap is homogeneously present as a monomeric form in solution, which adopts an extended and structured conformation (confirming our previous findings). When Eap is in complete occupancy with CG the structure remains in extended state similar to the unbound Eap. However, when NE is occupied at all its site the structure is no longer extended but adopts a more compact form. These observations show that the linker residues present between the Eap domains provide enough flexibility to the domains and do not restrict domains from binding NSPs to the adjacent domains.

Additionally, Eap domain 4 exclusively was found to be completely inhibiting NE even at sub-stoichiometric concentration. These observations implicated a plausible presence of more than one NE binding site at Eap4. Sec-SAXS analysis on Eap4 with 2-fold molar excess of NE resulted in a higher R_g , D_{max} , and molecular weight estimation compared to CG/EapH2/NE (which is present as a monomer in solution) indicative of a plausible dimer in solution. Crystal structure analysis of Eap4 with a 2-fold excess of NE not only confirmed the SAXS findings but also provided the atomic level understanding of NE inhibition at both the binding sites. The presence of a dimer of the Eap4/2NE complex in the ASU helped to solve the conundrum of the basis of dimerization of the complex. Given that the particle presents as a dimer in the solution, crystal structure aligned well with the SAXS profile resulting in an χ^2 of 1.41. The dimerization of the

dimer at the C-terminus of Eap4 directed us to further explore the possibility of a higher order structure in the Eap/NSP complex.

We further expanded our understanding of NSP inhibition by the Eap protein, providing evidence supporting polyvalent NSP inhibition and oligomerization of the NSP-occupied multidomain Eap. HT-SAXS analysis of NSP/Eap34 not only confirms the occupancy of all available NSP binding sites but also indicates orderly oligomerization of the complex through the C-terminal region of Eap domain 4. Although we were unable to generate SAXS data with NSP/Eap full-length, given the strong binding affinities between NSPs and Eap domains, Eap full-length can occupy all its binding sites with NSPs in solution while forming ordered oligomers (Fig. 7.7). Exploring this possibility will require NSP/Eap complex analysis using cryo-EM. However, the key question remains: how do bacteria benefit from this? Neutrophils use NETs to entrap and kill bacteria, placing a selective pressure on bacteria to overcome this defense mechanism. Eisenbeis et al. discovered Eap's ability to aggregate DNA and interfere with NET formation. We hypothesize that NSP/Eap oligomerization may prevent effective NET formation or render the NET backbone, DNA, more susceptible to lysis by bacterial DNase. This hypothesis also explains the presence of additional binding sites for NE. Owing to the slight excess concentration of NE compared to other NSPs in human neutrophil azurophilic granules (27) and indispensable role of NE in NETosis initiation (8), inhibiting NE preferentially over CG can hamper NET formation. Further explorations by performing cell-based assays using neutrophils to assess the degree of NETosis affected due to the presence of Eap is required to enhance our understanding of how *S. aureus* is employing Eap to dodge entrapment by the NET.

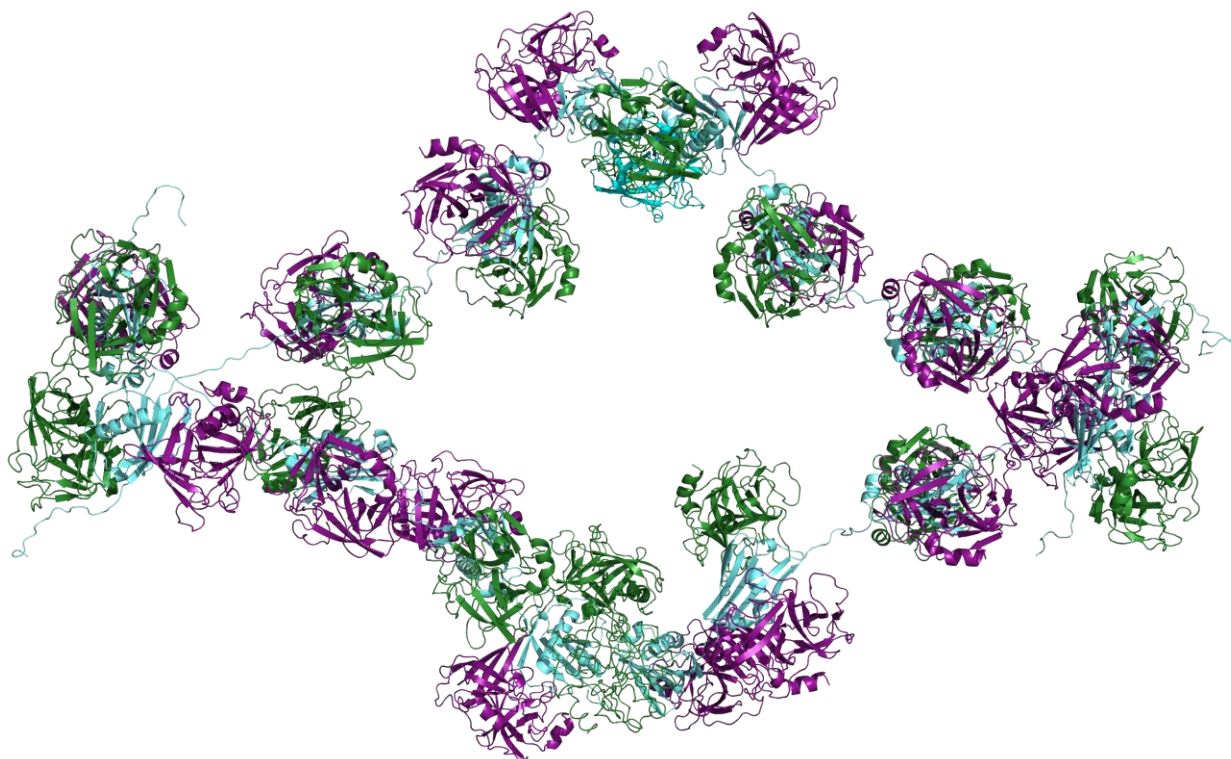


Figure 7.7 Hypothetical higher order structure of Eap in its complete occupation with NSPs.

HT-SAXS analysis of Eap34 revealed the existence of dimers in its complete NSP occupancy. Dimerization was also observed in CG/Eap4/NE and NE/Eap4/NE crystal structures. Moreover, the crystal structure of CG/Eap1/NE also provided evidence of dimerization occurring through the C-terminus of Eap1. Employing these findings and structural information, we constructed a hypothetical model for the full-length Eap bound to NSPs at all its sites. Cyan represents Eap, green represents NE and purple represents CG.

Materials and Methods

Protein crystallography and structural analysis

Protein single crystals or protein-protein complexes were acquired following the procedures outlined in the Supplemental Materials. Cryopreserved samples were subjected to monochromatic X-ray diffraction data collection at beamlines 22-BM and 22-ID located at the Advanced Photon Source of Argonne National Laboratory. Subsequently, X-ray diffraction data underwent indexing, integration, and scaling through a blend of HKL2000, XDS, and AIMLESS software packages. The structures were solved employing molecular replacement via PHASER (28) within the PHENIX suite (29), utilizing specific search models detailed in the Supplemental Materials for each target structure. Initial model solutions were improved through automated rebuilding using PHENIX.AUTOBUILD (29) or manual adjustment using COOT (30). Crystallographic models underwent refinement through a combination of methods including simulating annealing, reciprocal space positional refinement, B-factor refinement, and TLS refinement using PHENIX.REFINE (29). Refined models and corresponding structure factors have been deposited in the PDB, with accession codes provided in Table 7.1. Detailed information regarding diffraction data collection statistics and model properties for each structure is also available in Table 7.1.

Representations of protein structures were generated from the respective PDB files utilizing PyMol (Shrodinger, LLC) (31). The Root Mean Square Deviation (R.M.S.D.) of superposition for individual proteins or protein-protein complexes was calculated using PyMol. Buried surface area at interfaces, along with automated identification of hydrogen bonds and salt bridges, were determined using EBI-PISA (32). Surface complementarity between interacting proteins was assessed using the Sc program (33) within the CCP4 suite (34). Protein sequence

alignments and evaluations of sequence similarity/identity were conducted using the EMBL-EBI Clustal Omega suite (35).

Small-angle X-ray scattering experiments

We conducted small-angle X-ray scattering (SAXS) experiments at the SIBYLS beamline 12.3.1 of the Advanced Light Source. This beamline features inline instrumentation and detectors connected to a size exclusion column (36, 37). Samples of 5 mg/ml concentration were prepared in 20 mM Hepes (pH 7.4), 140 mM NaCl SEC running buffer (for EapH₂, CG, CG/EapH₂/NE, NE/Eap4/NE, Eap and Eap/4CG) and 20 mM Acetate (pH 4.0), L-Arginine 100 mM, L-Glutamic acid 100 mM, NaCl 300 mM, 1 % Glycerol (For Eap/4NE, Eap34, Eap34/2CG/2NE). The X-ray wavelength was set to 1.127 Å, and the sample-to-detector distance to 2,100 mm, resulting in scattering vectors (q) ranging from 0.01 Å⁻¹ to 0.4 Å⁻¹. The SAXS flow cell was connected to an Agilent 1260 Infinity HPLC system with a Shodex 803 SEC column at a flow rate of 0.65 ml/min. SAXS measurements involved continuous 2 sec X-ray exposures collected over a 25 min elution period, with frames analyzed after subtracting a SAXS frame corresponding to the running buffer before peak detection.

We utilized the Guinier approximation ($I(q) = I(0) \exp(-q^2 R_g^2/3)$, with $qR_g < 1.3$) to calculate the radius of gyration (R_g) for each subtracted frame. The elution peak was assessed by comparing the integral ratios to background and R_g relative to the recorded frame using RAW (38). Uniform R_g values across the elution peak indicate a homogeneous sample. Merged SAXS profiles, obtained by integrating multiple frames at the elution peak, were further analyzed. The Guinier plot provided insights into the aggregation state, while the volume of correlation (V_c)

estimated the molecular weight (39), and the pair distribution function $[P(r)]$ calculated the maximal inter-particle dimension (40).

HT-SAXS analysis for all the samples were conducted at three different concentrations. Eap34/2CG/2NE was analyzed using 20 mM Hepes (pH 7.4), 140 mM NaCl at 5, 2.5 and 1.25 mg/ml concentration. RAW, ATSAS and Scatter were used for data processing and BilboMD, multiFOXS and FOXS were used for model building and assessment.

Ensemble generation and optimization

The previously determined crystal structures of Eap domain proteins 1 to 4 in the presence of CG and NE served as input for the comparative modeler (41). To create a unified structure, the domains were linked using the comparative modeler program in Chimera-X. Multi-state modeling with SAXS profiles was then conducted using MultiFOXS (42), connecting all the chains as rigid bodies. A total of 10,000 conformations were generated, and SAXS profiles were calculated using FOXS (43). The conformations with lower X^2 values were selected for final assessments and subsequent submissions. BilboMD was also used in multistate modeling of Eap34/NSP (44).

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Supplemental Materials

Protein samples

Human neutrophil elastase was procured from Elastin Products Company, while human neutrophil cathepsin-G was obtained from Innovative Research. Derivatives of the prokaryotic expression vector pT7HMT (1) encoding either wild-type or mutant forms of Eap1, Eap2, Eap3, Eap4, EapH1, Eap and Eap34 were obtained from GenScript USA Inc. Both mutant and wild-type Eap proteins were expressed from plasmid using *E. coli* BL21(DE3) cells and purified as previously described elsewhere (2, 3). The concentrations of wild-type and mutant Eap proteins were assessed spectrophotometrically using a DS-11 Spectrophotometer from DeNovix using the extinction coefficient derived from the deduced protein composition. (EapH1: $\epsilon_{280}=7.45 \text{ mM}^{-1}\text{cm}^{-1}$; EapH2: $\epsilon_{280}=14.44 \text{ mM}^{-1}\text{cm}^{-1}$; Eap1: $\epsilon_{280}=7.45 \text{ mM}^{-1}\text{cm}^{-1}$; Eap2: $\epsilon_{280}=5.96 \text{ mM}^{-1}\text{cm}^{-1}$, Eap3: $\epsilon_{280}=15.93 \text{ mM}^{-1}\text{cm}^{-1}$, Eap4: $\epsilon_{280}=8.94 \text{ mM}^{-1}\text{cm}^{-1}$, Eap: $\epsilon_{280}=41.26 \text{ mM}^{-1}\text{cm}^{-1}$, Eap34: $\epsilon_{280}=26.36 \text{ mM}^{-1}\text{cm}^{-1}$) ExPASy suite ProtParam (4, 5).

Preparation of neutrophil elastase, cathepsin-G, and substrates

Lyophilized human neutrophil elastase (1 mg) was dissolved in 200 μL of 20 mM sodium acetate/50% glycerol (pH: 5.0), stored at -20°C in 10 μL aliquots. Enzyme concentration was determined using the protein composition ($\epsilon_{280}=20.23 \text{ mM}^{-1}\text{cm}^{-1}$). The NE substrate, methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanalide (MSA2PV-pNA) (from Sigma-Aldrich) was dissolved in 100% (v/v) DMSO, was diluted in 50 mM HEPES/140 mM NaCl/0.05% Tween-20 (pH: 7.4) to achieve the working concentration and quantified using end-point assays ($\epsilon_{400}=12.3 \text{ mM}^{-1}\text{cm}^{-1}$). Human cathepsin-G was stored at 4°C in 50 mM sodium acetate/800 mM NaCl (pH 5.5) and diluted prior to use using buffer 20 mM HEPES/140 mM NaCl/0.05% (v/v) Tween-20

(pH: 7.4). Succinyl-Ala-Ala-Pro-Phe-p-nitroanalide (SA2PF-pNA) (from Kayman Chemical Company) was dissolved in 100% (v/v) DMSO and stored with desiccant. The fluorescent substrate, methoxy succinyl-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin (MSA2PV-AMC) (from Cayman Chemical Company), was prepared in 100% (v/v) DMSO (for the final concentration of 15.9 mM).

Protein crystallography and structural analysis

Crystallization samples of individual proteins were prepared according to the above-outlined procedure. In cases involving protein complexes, equimolar ratios of the required monomers were mixed for reconstitution. The ternary complexes of Eap1 and Eap2 were buffer exchanged into the buffer 10 mM HEPES/50 mM NaCl (pH: 7.4), resulting in a concentration of 10 mg/ml. For other complexes, the buffer exchange was carried out into 10 mM Tris/50 mM NaCl (pH: 7.4), achieving a final concentration of 5 mg/ml. The concentration of each sample was determined based on the calculated extinction coefficient of the respective complexes. Initial crystallization conditions were determined using a vapor diffusion sitting drops method with Crystal Gryphon liquid handling system (Art Robbins Instruments, LLC). The Crystal Screen HT, Index HT, and PEGRx HT screening matrices from Hampton Research Corp. were used in this screening process. Screening droplets, comprising a total volume of 0.4 μ L, were prepared in both 3:1 and 2:2 ratios of sample to precipitant solution and equilibrated alongside 100 μ L of precipitant solution. Optimization of individual crystallization conditions was carried out through vapor diffusion hanging drops, involving systematic adjustments to sample concentration, buffer pH, precipitant concentration, and potential utilization of microseeding techniques. All initial crystallization screens and optimization studies were conducted at a temperature of 20 °C. Further

details regarding the ultimate crystallization conditions and the composition of individual models are elaborated in subsequent paragraphs.

Crystals of the ternary complex of Eap1 bound to CG and NE were obtained from a hanging drop that had been prepared by mixing 1 μ L of 5 mg/ml protein with 1 μ L of a precipitant solution consisting of 0.1 M BIS-TRIS, 0.2 M sodium/potassium tartrate tetrahydrate, 14% (w/v) PEG-8000 (pH: 6.9). Individual crystals were cryoprotected prior to harvest by briefly soaking in a buffer of precipitant solution supplemented with 11% (v/v) glycerol. Monochromatic X-ray diffraction data extending to 3.63 Å limiting resolution were collected using APS beamline 22-ID and were indexed, integrated, and scaled in the space group $P2_12_12_1$ using XDS and AIMLESS. The structure was solved by molecular replacement using the structures of Eap1 (PDB entry 8D4O), CG (PDB entry 1AU8), and NE (PDB entry 1NE) as search models and contains six ternary complexes in the asymmetric unit. The final model consists of six complete copies of Eap1, six complete copies of CG, and six complete copies of NE. Using the complex defined by Chains A, B, and C as a basis for comparison, the remaining complexes superimpose with RMSD values of 0.282 Å (Chains D, E, and F), 0.336 Å (Chains G, H, and I), 0.512 Å (Chains J, K, and L), 0.408 Å (Chains M, N, and O), and 0.584 Å (Chains P, Q, and R), respectively. The final model and refined structure factors have been deposited in the PDB as entry 8D7I.

Crystals of the ternary complex of Eap2 bound to CG and NE were obtained from a hanging drop which was prepared by mixing 1 μ L of 5 mg/ml protein with 1 μ L of a precipitant solution consisting of 0.1 M citric acid, 0.15 M lithium sulfate monohydrate, 12% (w/v) PEG-6000 (pH: 3.6). Individual crystals were cryoprotected prior to harvest by briefly soaking in a buffer of precipitant solution supplemented with 23% (w/v) PEG-6000. Monochromatic X-ray diffraction data extending to 3.10 Å limiting resolution were collected using APS beamline 22-ID and were

indexed, integrated, and scaled in the space group $P2_1$ using HKL2000. The structure was solved by molecular replacement using the structures of Eap2 (PDB entry 1YN3), CG (PDB entry 1AU8), and NE (PDB entry 1NE) as search models and contains four ternary complexes in the ASU. The final model consists of four complete copies of Eap2, four complete copies of CG, and four complete copies of NE. Using the complex defined by Chains A, B, and C as a basis for comparison, the remaining complexes superimpose with RMSD values of 0.269 Å (Chains D, E, and F), 0.625 Å (Chains G, H, and I), and 0.367 Å (Chains J, K, and L), respectively. The final model and refined structure factors have been deposited in the PDB as entry 8D7K.

Crystals of the ternary complex of Eap3 with CG and NE were grown using a hanging drop method, where 3 µL of 5 mg/ml protein was mixed with 1 µL of a precipitant solution containing 0.2 M ammonium citrate, 0.1 M imidazole (pH 6.8), and 21% (w/v) peg-2K-MME. Prior to harvesting, individual crystals were briefly soaked in a solution containing 14% (v/v) glycerol for cryoprotection. X-ray diffraction data up to 1.95 Å resolution were collected at APS beamline 22-ID, indexed, integrated, and scaled in space group $P212121$ using HKL2000. Molecular replacement, utilizing structures of Eap2 (PDB: 1YN3), CG (PDB: 1AU8), and NE (PDB: 1NE), was employed to solve the structure, revealing a single ternary complex in the ASU. The final refined model and structure factors have been deposited in the PDB under entry 9ASX.

The binary complex crystal structure for Eap4/NE was obtained via the sitting drop method, where 5 mg/ml protein was mixed with a precipitant solution consisting of 10 mM Zinc sulfate heptahydrate, 0.1 M MES pH 6.5, and 25% peg-550MME in a 3:1 ratio. Individual crystals were cryoprotected by briefly soaking them in a buffer of precipitant solution supplemented with 10% peg-400. The diffraction data with 1.75 Å resolution limit were collected at APS beamline 22-ID and processed in space group $F222$ using HKL2000. The structure was solved by molecular

replacement using Eap2 (PDB entry 1YN3) and NE (PDB entry 1NE) as search models, revealing one binary complex in the ASU. The refined structure has been deposited in the PDB as entry 9ASS. Additionally, the Eap4 model from the Eap4/NE complex was utilized as a search model during molecular replacement for CG/Eap4/NE and NE/Eap4/NE crystal structures.

The CG/Eap4/NE ternary crystal structure was obtained via the hanging drop method, mixing 3 μ L of 5 mg/ml protein with 1 μ L of a precipitant solution containing 0.1 M sodium citrate (pH 5.5) and 12% (w/v) PEG-6K. Individual crystals were cryoprotected by brief soaking in a buffer of precipitant solution supplemented with 23% (v/v) MPD. The x-ray diffraction data with 2.10 Å limiting resolution were collected at APS beamline 22-ID, processed in space group P1 using HKL2000 software. Molecular replacement, using Eap4 (PDB entry 9ASS), CG (PDB entry 1AU8), and NE (PDB entry 1NE) as search models, revealed four ternary complexes in the ASU i.e. two dimer complexes. The refined structure has been deposited in the PDB as entry 9ATK.

Crystals of the NE/Eap4/NE ternary complex were obtained via the hanging drop method by mixing 3 μ L of 5 mg/ml protein with 1 μ L of a precipitant solution containing 0.2 M NH₄Ac, 0.1 M TRIS-Cl (pH 8.0), and 17% PEG 10K. Individual crystals were cryoprotected by brief soaking in a buffer of precipitant solution supplemented with 18% (v/v) glycerol. X-ray diffraction data extending to 2.05 Å resolution were collected at APS beamline 22-ID, processed in space group P212121 using HKL2000. Molecular replacement, utilizing Eap4 (PDB entry 9ASS) and NE (PDB entry 1NE) as search models, revealed two ternary complexes in the ASU. The refined structure has been deposited in the PDB as entry 9ATU.

Simultaneous inhibition of human neutrophil elastase and human cathepsin-G by Eap proteins

To probe the ability of the EAP-domain proteins to inhibit both neutrophil serine proteases simultaneously, parallel reaction pairs were prepared consisting of both proteases with their respective substrates with or without the EAP-domain inhibitor. To investigate simultaneous inhibition by Eap1, four separate pairs of reactions were prepared. Reaction pair one consisted of 15 nM CG with 340 μ M MSA₂PV-AMC in the top well and 15.2 nM NE with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair two consisted of 15 nM CG with 340 μ M MSA₂PV-AMC and 18.8 nM Eap1 in the top well with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair three consisted of 15 nM CG with 340 μ M MSA₂PV-AMC and 18.8 nM Eap1 in the top well and 15.2 nM NE with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair four consisted of 340 μ M MSA₂PV-AMC with 18.8 nM Eap1 in the top well and 15.2 nM NE with 2.35 mM SA₂PF-pNA in the lower well. The reactions were initiated by mixing 170 μ L of the top well into 170 μ L of the lower well. The final concentrations of the reaction components were 7.5nM CG, 7.6 nM NE, 9.4 nM Eap1, 170 μ M MSA₂PV-AMC and 1.175 mM SA₂PF-pNA.

To investigate simultaneous inhibition by Eap2, four separate pairs of reactions were also prepared. Reaction pair one consisted of 16.4 nM CG with 340 μ M MSA₂PV-AMC in the top well and 14.6 nM NE with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair two consisted of 16.4 nM CG with 340 μ M MSA₂PV-AMC in the top well and 20.6 nM Eap2 with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair three consisted of 16.4 nM CG with 340 μ M MSA₂PV-AMC in the top well and 14.6 nM NE with 2.35 mM SA₂PF-pNA and 20.6 nM Eap2 in the lower well. Reaction pair four consisted of 340 μ M MSA₂PV-AMC in the top well and 14.6 nM NE with 2.35 mM SA₂PF-pNA and 20.6 nM Eap2 in the lower well. The reactions were initiated by mixing 170

μL of the top well into 170 μL of the lower well. The final concentrations of the reaction components were 8.2 nM CG, 7.3 nM NE, 10.3 nM Eap2, 170 μM MSA₂PV-AMC and 1.175 mM SA₂PF-pNA.

The NE reaction was followed fluorometrically (λ_{ex} : 355 nm, λ_{em} : 455 nm) while the CG reaction was followed spectrophotometrically (400 nm). The buffer consisted of 50 mM HEPES/140 mM NaCl/0.05% (v/v) tween-20/5% (v/v) DMSO (pH: 7.4).

Simultaneous inhibition of neutrophil elastase and cathepsin-G by EAP domain proteins post 30 minute pre-incubation

The simultaneous inhibition of CG and NE by Eap1 or Eap2 was performed as stated above, apart from allowing the reaction components to incubate 30 minutes prior to mixing. A similar strategy was used to investigate simultaneous inhibition by EapH1. Reaction pair one consisted of 15 nM CG incubated with 340 μM MSA₂PV-AMC in the top well with 40.4 nM NE incubated with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair two consisted of 15 nM CG incubated with 340 μM MSA₂PV-AMC in the top well with 39 nM EapH1 incubated with 2.35 mM SA₂PF-pNA in the lower well. Reaction pair three consisted of 15 nM CG incubated with 340 μM MSA₂PV-AMC in the top well with 40.4 nM NE incubated with 2.35 mM SA₂PF-pNA and 39 nM EapH1 in the lower well. Reaction pair four consisted of 340 μM MSA₂PV-AMC in the top well with 40.4 nM NE incubated with 2.35 mM SA₂PF-pNA and 39 nM EapH1 in the lower well. The reaction components were allowed to incubate for 30 minutes prior to mixing. The final concentrations of the reaction components were 7.5 nM CG, 20.2 nM NE, 19.5 nM EapH1, 170 μM MSA₂PV-AMC and 1.175 mM SA₂PF-pNA.

The NE reaction was followed fluorometrically (λ_{ex} : 355 nm, λ_{em} : 455 nm) while the CG reaction was followed spectrophotometrically (400 nm). The buffer consisted of 50 mM HEPES/140 mM NaCl/0.05% (v/v) tween-20/5% (v/v) DMSO (pH: 7.4).

Fig. S1

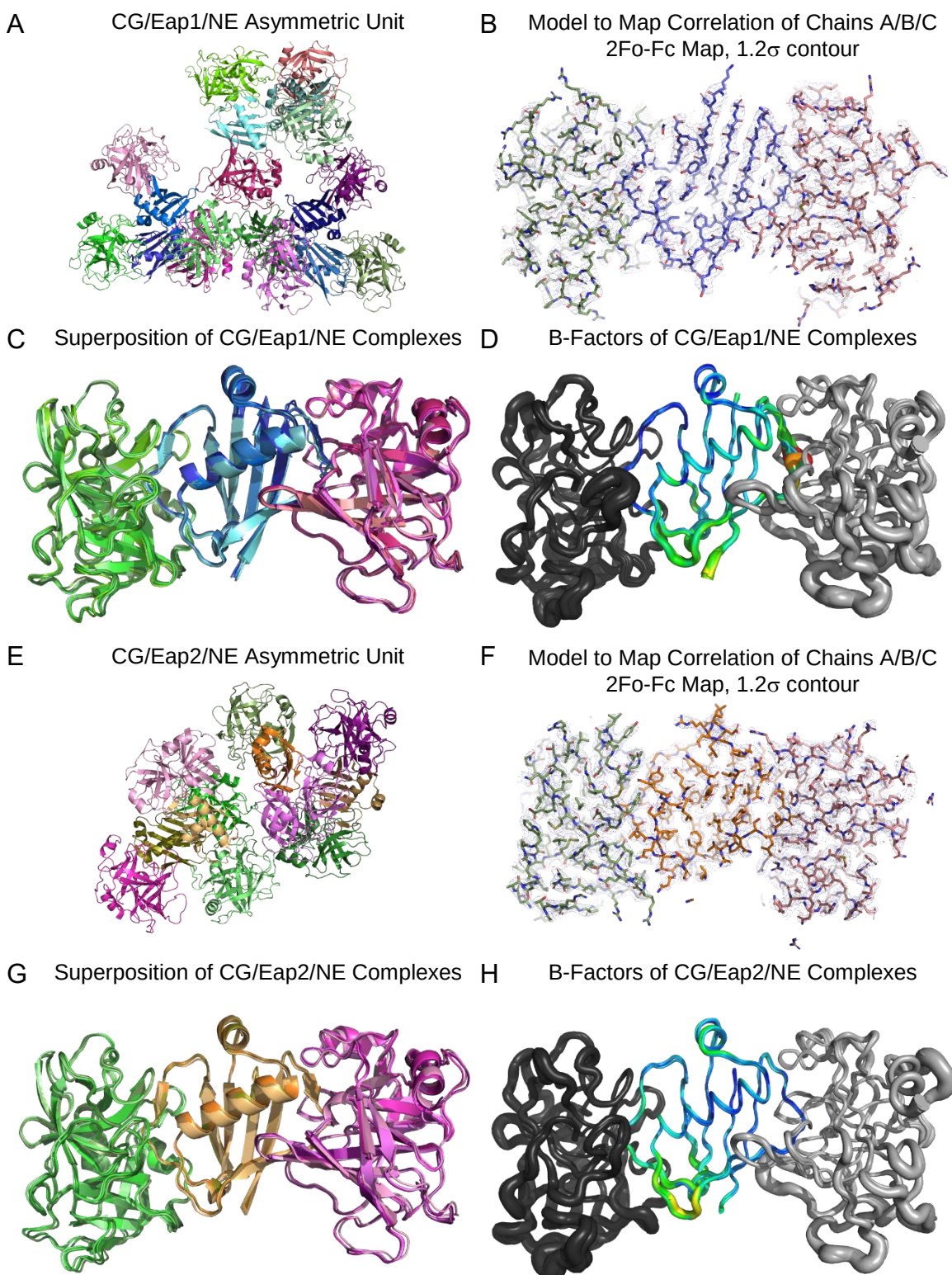
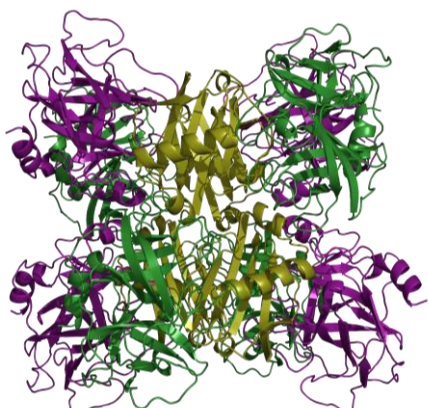


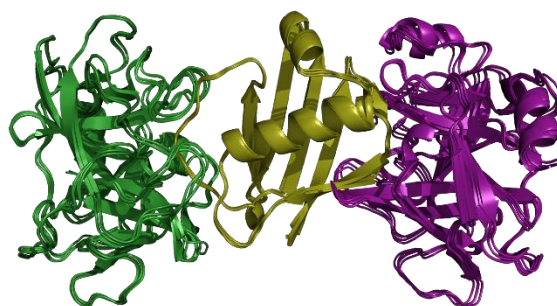
Figure S1. Supporting data pertaining to the CG/Eap1/NE and CG/Eap2/NE crystal structures. (A) Representation of the complete asymmetric unit for the crystal structure of CG/Eap1/NE. The final model consists of six copies of the CG/Eap1/NE complex. CG molecules are colored in shades of purple, NE molecules are colored in shades of green, and Eap1 molecules are colored in shades of blue. (B) Representative model to map correlation for the CG/Eap1/NE structure. A region corresponding to the protein-protein interface between Chain A (NE), Chain B (Eap1), and Chain C (CG) of the final model is drawn in ball-and-stick convention with carbon atoms colored green, blue, and light purple, respectively. The 3.63 Å resolution 2Fo-Fc electron density map contoured at 1.2σ is shown as a grey mesh. (C) Superposition of the six complexes found in the asymmetric unit of the CG/Eap1/NE crystal. Proteins are drawn as ribbon diagrams with CG (purple), Eap1 (blue), and NE (green) polypeptides colored identically to panel A. (D) Representation of the six superimposed CG/Eap1/NE complexes colored according to their B-factors in the final model. Positions of low B-factors appear thinner and are further colored blue for the inhibitor, while those with high B-factors appear thicker and are further colored red for the inhibitor. (E) Representation of the entire asymmetric unit for the crystal structure of CG/Eap2/NE. The final model comprises of four copies of the CG/Eap2/NE complex. CG molecules are colored in shades of purple, NE molecules are colored in shades of green, while Eap2 molecules are colored in shades of orange. (F) Representative model to map correlation for the CG/Eap2/NE structure. A region corresponding to the protein-protein interface between Chain A (NE), Chain B (Eap2), and Chain C (CG) of the final model is drawn in ball-and-stick convention with carbon atoms colored green, orange, and light purple, respectively. The 3.1 Å resolution 2Fo-Fc electron density map contoured at 1.2σ is shown as a grey mesh. (G) Superposition of the four complexes found in the asymmetric unit of the CG/Eap2/NE crystal. Proteins are drawn as ribbon diagrams with CG (purple), Eap2 (orange), and NE (green) colored identically to panel E. (H) Representation of the four superimposed CG/Eap2/NE complexes colored according to their B-factors in the final model. Positions of low B-factors appear thinner and are further colored blue for the inhibitor, while those with high B-factors appear thicker and are further colored red for the inhibitor.

Fig. S2

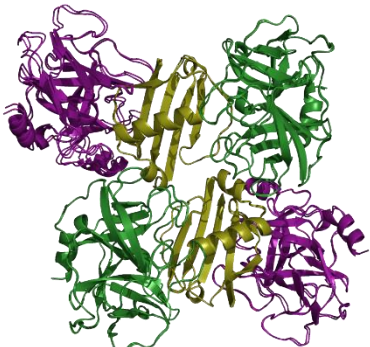
A. CG/Eap4/NE ASU



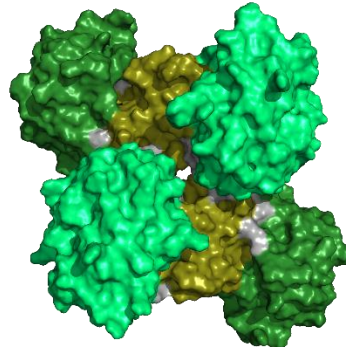
B. Superposition of CG/Eap4/NE complexes



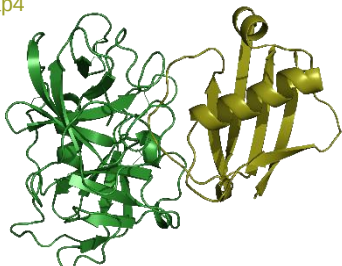
C. CG/Eap4/NE dimer overlaid



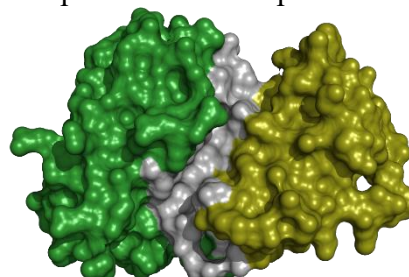
D. Eap4/2NE surface representation



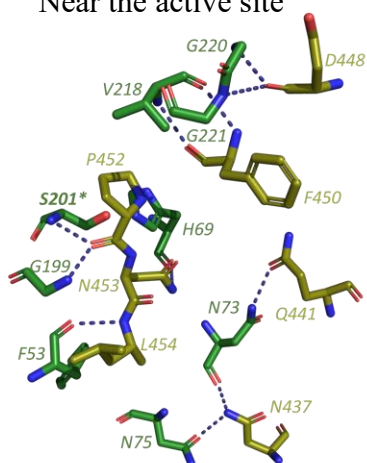
E. NE Eap4/NE binary complex
Eap4



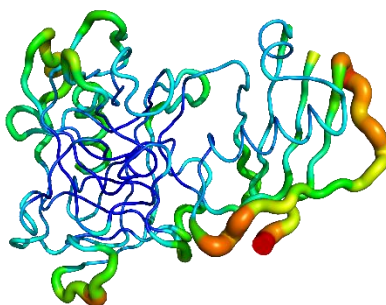
F. Eap4/NE surface representation



G. Eap4/NE interactions
Near the active site



H. Eap4/NE B-factor



I. NE/Eap4/NE B-factor

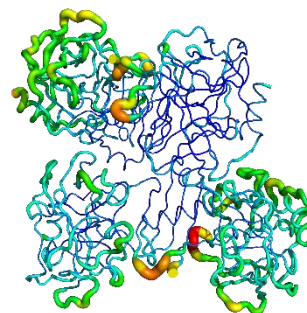


Figure S2. Supporting data for ternary complexes of Eap3 and Eap4. (A) Representation for CG/Eap4/NE ternary complexes in the ASU. It shows the presence of two copies of the dimer ternary complex. NE is in green, CG is in purple and Eap4 is in olive color. (B) Overlay of all the Eap4 ternary complex structures from ASU. Green represents NE, purple is for CG and Eap4 is in olive color. (C) Superposition of the two CG/Eap4/NE dimer complexes. The complexes form via the interactions through the Eap4 C-terminal region. (D) Surface representation of the dimer of NE/Eap4/NE ternary complex. Green represents the NE bound to Eap4 at the conserved site while light green represents the NE bound to the alternate Eap4 site. Eap4 is represented in olive color while the interacting sites are shown in grey. (E) Cartoon representation of Eap4/NE binary complex. Eap4 is shown in olive while NE is in green. (F) Surface representation of Eap4/NE binary complex to represent interactions present. Olive color corresponds to Eap4, green represents NE, and grey shows the interactions present between the proteins. (G) The Eap4/NE binary complex is shown as a stick model to represent the interactions between NE and Eap4. The hydrogen bonds are presented as blue dotted lines. (H) B-factor distribution for Eap4/NE binary complex (Blue represents the lower B-factor while red is for higher). (I) B-factor distribution for NE/Eap4/NE ternary complex.

Fig. S3

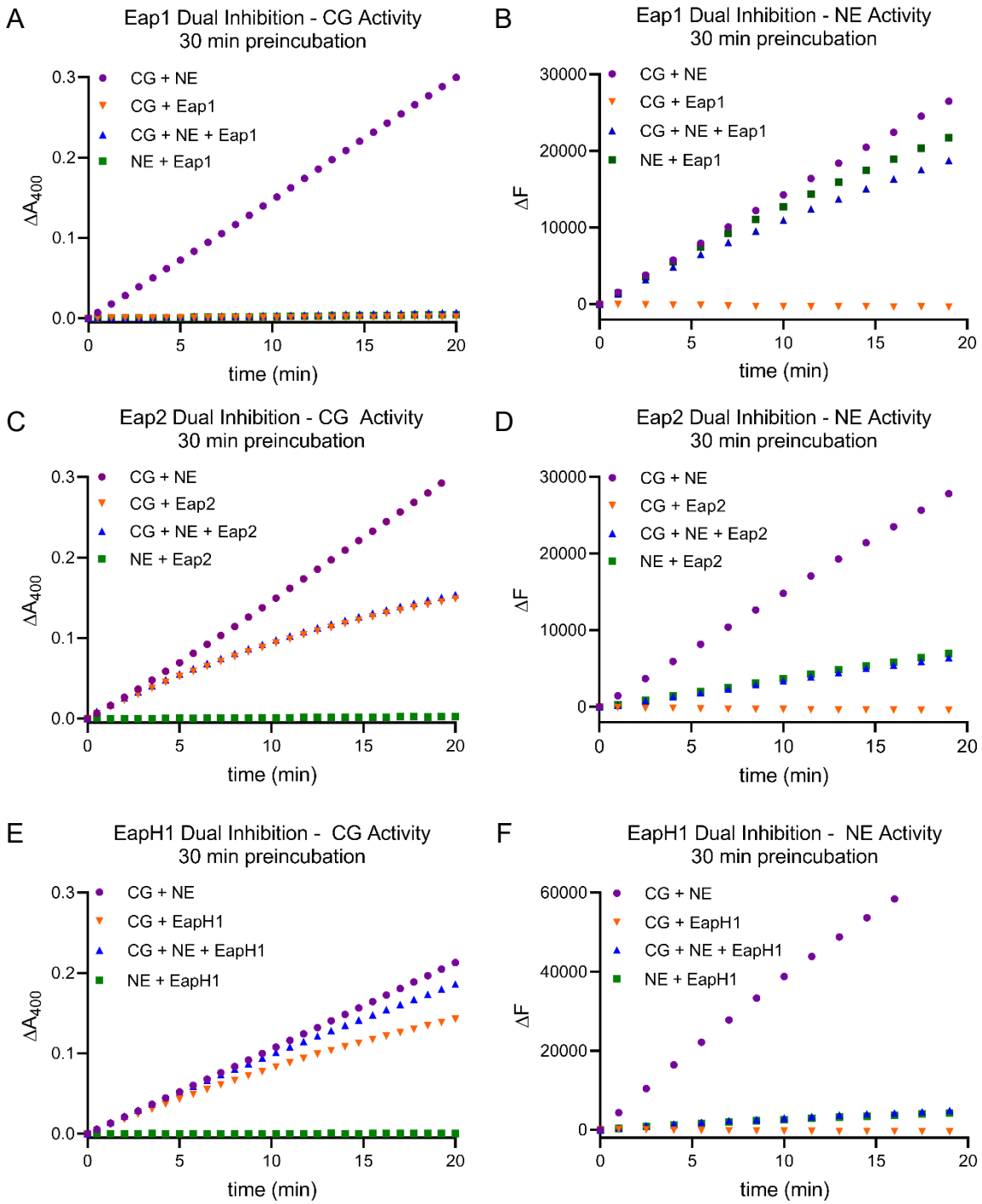


Figure S3. Supporting data pertaining to the simultaneous inhibition of CG and NE by Eap1, Eap2 and EapH1 following a 30 minute pre-incubation of NSPs and EAP proteins. (A) Simultaneous inhibition of CG and NE by Eap1 followed by SA₂PF-pNA turnover by CG. (●) Reaction consists of 7.5 nM CG, 7.6 nM NE, 0 nM Eap1. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 9.4 nM Eap1. (▲) Reaction consists of 7.5 nM CG, 7.6 nM NE, 9.4 nM Eap1. (■) Reaction consists of 0 nM CG, 7.6 nM NE, 9.4 nM Eap1. (B) Simultaneous inhibition of CG and NE by Eap1 followed by MSA₂PV-AMC turnover by NE. (●) Reaction consists of 7.5 nM CG, 7.6 nM NE, 0 nM Eap1. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 9.4 nM Eap1. (▲) Reaction consists of 7.5 nM CG, 7.6 nM NE, 9.4 nM Eap1. (■) Reaction consists of 0 nM CG, 7.6 nM NE, 9.4 nM Eap1. (C) Simultaneous inhibition of CG and NE by Eap2 followed by SA₂PF-pNA turnover by CG. (●) Reaction consists of 8.2 nM CG, 7.3 nM NE, 0 nM Eap2. (▼) Reaction consists of 8.2 nM CG, 0 nM NE, 10.3 nM Eap2. (▲) Reaction consists of 8.2 nM CG, 7.3 nM NE, 10.3 nM Eap2. (■) Reaction consists of 0 nM CG, 7.3 nM NE, 10.3 nM Eap2. (D) Simultaneous inhibition of CG and NE by Eap2 followed by MSA₂PV-AMC turnover by NE. (●) Reaction consists of 8.2 nM CG, 7.3 nM NE, 0 nM Eap2. (▼) Reaction consists of 8.2 nM CG, 0 nM NE, 10.3 nM Eap2. (▲) Reaction consists of 8.2 nM CG, 7.3 nM NE, 10.3 nM Eap2. (■) Reaction consists of 0 nM CG, 7.3 nM NE, 10.3 nM Eap2. (E) Simultaneous inhibition of CG and NE by EapH1 followed by SA₂PF-pNA turnover by CG. (●) Reaction consists of 7.5 nM CG, 20.2 nM NE, 0 nM EapH1. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 19.5 nM EapH1. (▲) Reaction consists of 7.5 nM CG, 20.2 nM NE, 19.5 nM EapH1. (■) Reaction consists of 0 nM CG, 20.2 nM NE, 19.5 nM EapH1. (F) Simultaneous inhibition of CG and NE by EapH1 followed by MSA₂PV-AMC turnover by NE. (●) Reaction consists of 7.5 nM CG, 7.3 nM NE, 0 nM EapH1. (▼) Reaction consists of 7.5 nM CG, 0 nM NE, 19.5 nM EapH1. (▲) Reaction consists of 7.5 nM CG, 20.2 nM NE, 19.5 nM EapH1. (■) Reaction consists of 0 nM CG, 20.2 nM NE, 19.5 nM EapH1. Substrate concentrations in all reactions were 1.175 mM SA₂PF-pNA and 170 μ M MSA₂PV-AMC.

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Chapter 8 - Conclusions

S. aureus is a widespread commensal bacterium found in about one-third of the human population, yet it poses a significant health threat and causes various infections ranging from superficial skin diseases to severe conditions like bacteremia, sepsis, and pneumonia (1, 2). Previous work has shown that *S. aureus* adeptly evades host immune defenses through a myriad of immune evasion proteins, many of which target neutrophils (3–5). Neutrophils employ potent mechanisms within their phagosomes to eliminate bacteria such as *S. aureus*, including myeloperoxidase (MPO)-mediated oxidative killing and serine proteases capable of degrading bacterial proteins. However, *S. aureus* has evolved strategies to counteract these cytotoxic components. For instance, it produces the staphylococcal peroxidase inhibitor (SPIN) to block the production of hypohalous acids by MPO and the extracellular adherence protein (Eap) to bind neutrophil serine proteases (NSPs), thus blocking their activity (3, 5). This dissertation focused on unraveling the structure-function relationships of two families of staphylococcal immune evasion proteins and their complexes with key enzymes within the neutrophil antibacterial arsenal. Therefore, this work has enhanced our molecular-level understanding of how *Staphylococcus aureus* circumvents the human innate immune response.

Analysis of the SPIN/MPO co-crystal structure revealed that SPIN binds to MPO near its active site, effectively obstructing access of the initial substrate, H_2O_2 , to the catalytic heme. The bound SPIN adopts a triple helical structure responsible for MPO binding, along with an N-terminal region of approximately ten residues that undergoes a structural transformation into a β -hairpin-like conformation upon binding to MPO (3); this N-terminal region remains disordered in the absence of MPO (6). Introducing a disulfide bond to physically constrain the SPIN N-terminal loop region resulted in enhanced N-terminal stability compared to wild-type SPIN, as judged by

NMR relaxation approaches. The solution NMR structure displayed a characteristic triple helical structure at the C-terminus of SPIN-cys while the N-terminus remained disordered yet constrained. SPR analysis showed that introducing a disulfide at the SPIN N-terminus enhanced the affinity of SPIN towards MPO by 2.5-fold. Mirroring the SPR results, SPIN-cys inhibited MPO more potently than the wild-type counterpart. Our interpretation of these data is that the primary factor driving the enhancement of affinity and inhibition is the restriction of conformational flexibility, arising from the disulfide, during the interaction between SPIN and MPO.

S. aureus produces three proteins within the Eap family: the multidomain Eap protein and two single domain Eap homologs known as EapH1 and EapH2. While the interaction between NSPs and EapH1 has been extensively studied and serves as a model for understanding NSP inhibition by Eap proteins, knowledge of the structural basis for EapH2/NSP interaction has been hampered by the absence of co-crystal structures (5). By employing NMR and crystallography techniques, we discovered a distinct NSP inhibitory mechanism for EapH2 when compared to EapH1. Contrary to EapH1, EapH2 possesses separate binding sites for CG and NE, enabling it to simultaneously neutralize both NSPs (Fig. 6.4) (5, 7). Enzyme inhibition assays corroborated this discovery. Together, this demonstrated that EapH2 bound to one NSP does not impede the binding of the other, indicating distinct binding sites on EapH2 for each NSP (Fig. 6.5). Analysis of the ternary crystal structure of CG/EapH2/NE revealed interactions between EapH2 and the catalytic serine of both NSPs, effectively blocking substrate access to both enzymes (7).

Thoroughly investigating the individual Eap domains and full-length Eap itself has further elucidated the structural underpinnings of NSP inhibition. Analysis of ternary complex structures revealed that all four domains inhibit NE in a manner like EapH2 (Fig. 7.1 & 7.4). Additionally, SAXS analysis revealed that full-length Eap has the capacity to simultaneously bind four

molecules of CG or NE, suggesting it can effectively target NSPs at all binding sites in solution (Fig. 7.5). Further exploration of NSP binding in the presence of Eap34 demonstrated that all four sites can be occupied by NSPs in solution (Fig. 7.6). Moreover, oligomerization via the C-terminal region of Eap4 suggests a potential presence of a higher order structure in full-length Eap when occupied by NSPs. Notably, Eap4 was found to uniquely bind two molecules of NE. Considering the indispensable role of NE in NET initiation (8), the occupation of both binding sites by NE could potentially provide bacteria a considerable advantage by thwarting NET initiation and preventing neutrophils from undergoing NETosis. (Fig. 7.4). Investigating how NET formation is impacted in the presence of Eap in future projects will serve to test this hypothesis. Additionally, plans to conduct cryo-EM studies to explore the full-length Eap bound with NSPs at all its sites are underway.

The findings presented in this dissertation have significantly enhanced our understanding of two families of immune evasion molecules produced by *S. aureus*. We explored the structure-function properties of a physically constrained SPIN N-terminus, which highlighted the role of N-terminal conformational flexibility in binding and inhibiting MPO. Further investigations using site-directed mutagenesis of the key residues in the SPIN N-terminal region can contribute to a deeper understanding of residue level interactions between these N-terminal residues and this critical enzyme within the neutrophil's oxidative defense systems. We also reported the first known example of bifunctional and simultaneous inhibition of two different serine proteases by EapH2. Subsequent structure/function characterization of the individual domains that comprise full-length Eap revealed that each of these domains displays an EapH2-like ability to bifunctionally inhibit CG and NE. Solution structure characterization of the two-domain variant, Eap34, in its complete NSP occupancy strongly suggests that Eap can bind NSPs simultaneously at all available

binding sites. It also hints at the possibility of oligomerization of NSP-bound Eap. Together, these outcomes have fundamentally changed our understanding of the molecular mechanisms used by *S. aureus* to survive inside neutrophil phagosome.

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Chapter 3



Staphylococcal peroxidase inhibitor (SPIN): Investigation of the inhibitory N-terminal domain via a stabilizing disulfide insertion

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Chapter 6



Simultaneous inhibition of two neutrophil serine proteases by the *S. aureus* innate immune evasion protein EapH2

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